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FOREWORD

The Library of The Marine Biological Laboratory is represented in this serial list, not only as four floors of journals and books reporting most of the scientific efforts of the world, but as a pleasant place in which to work, with unlimited facilities at one's fingertips. It is open to the use of everyone in attendance at the Laboratory and a limited number of readers may be accommodated with desk space upon approval of the Director, and payment of a fee.

A staff of librarians is in attendance, but it is felt that maximum value is obtained from the Library by free access to the stacks. Numerous tables give ample opportunity for work in close proximity to the collections. A large reading room with extensive racks and shelves for current journals, encyclopedias, maps, indexes and new books is provided with unlimited reading space.

The journals are arranged, beginning with the top floor, alphabetically as they appear in this list. Here they are listed under every possible title, and cross-referenced, and it is hoped that this revised edition of the library holdings will prove invaluable to investigators.

On the first level of the stacks a very large collection of separata, reprints and duplicate volumes is maintained. This is arranged in alphabetical order by authors, and these reprints may be charged out for use in the individual laboratory rooms on the cards provided at the main desk.

Reports of scientific expeditions published independently, and volumes of non-serial literature are shelved on the second stack level near the entrance from the reading room. These may be charged out in the same manner as reprints.

The Library has previously co-operated in granting Inter-library loans, but it is proposed now to reduce this as much as possible. There has been installed a microfilm apparatus suitable for copying from volumes of the usual quarto and octavo sizes. Copies of publications will be made upon the request of qualified individuals, at the lowest practicable rates. Information may be secured from the Librarian, and orders should be directed to her.

CHARLES PACKARD, *Director of the Laboratory*

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It is with deep regret that the Biological Bulletin
records the death, on January 4, 1943, of
Dr. Gary Nathan Calkins, a member of
the Editorial Board since 1927.

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THE
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PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

TRANSPLANTATION AND INDIVIDUALITY DIFFERENTIALS IN INBRED STRAINS OF RATS

LEO LOEB, H. D. KING, AND H. T. BLUMENTHAL

(*From the Laboratory of Research Pathology,* Oscar Johnson Institute, Washington University School of Medicine, St. Louis, and from the Wistar Institute, Philadelphia*)

In two previous papers (1927, 1931), Loeb and King have reported on the individuality differentials of rats inbred in the Wistar Institute for a considerable number of years by Dr. Helen Dean King. In this paper we shall report on a third series of experiments, carried out during the last two or three years, in which rats from inbred generations 102 to 106 were used. There was added, also, a small series of experiments with rats from inbred generations 91 and 92. However, in order to evaluate the changes which have taken place in the course of the continued inbreeding it will be necessary to give also the main data obtained in the first and second series of transplantations made with the earlier inbred generations. A comparison can be readily made if instead of discussing isolated data, we group experiments of a similar kind together by adopting a system of grades and determining the average grades obtained in each case. We have already adopted, in previous papers, such a system of approximately quantitative grading of the findings, and we have discussed these grades more fully on former occasions. Here, the principles underlying the choice of these grades will be only briefly explained. To simplify this task, we shall consider mainly the results obtained in transplantation of thyroid, cartilage, fat tissue, and also of the other organs often associated with these, namely, parathyroid, bone, bone marrow, striated muscle. It will not be necessary at this time to enter into a discussion of the corresponding reactions shown in all types of tissues used in our grafting experiments. The grades used range between 1 and 3+ (3.25); 3+ and 3 are the grades characteristic of autogenous transplantations. In this latter, the tissue—in particular, thyroid gland—is well preserved, and while at first there may be some irregularities in the structure of the graft, it gradually assumes the structure of the normal organ. Marked lymphocytic infiltration is lacking, but at early periods some very small collections of lymphocytes may be seen; subsequently, these usually disappear. Likewise, the connective tissue ingrowth is restricted and an invasion of the fat tissue by small vacuolated cells and by fibrous tissue is absent. Grades 3− (2.75) and 2+ (2.25) are given if the tissues are, on the whole, well preserved but if a reaction is definitely noticeable. Various degrees of lymphocytic infiltration and a somewhat increased activity of the connective tissue may appear and cause a limited injury to the transplant. Such reactions may be seen when donor and host are related. If these reactions are more marked and lead to a partial destruction of the transplant, the grade 2 is given; this indicates a somewhat greater strangeness of the -individuality differentials. In typical, more severe homoio-reactions the grades range between 2− (1.75) and 1. Grade 2− is given if the thyroid gland is severely invaded by fibrous tissue and a considerable part of the acinar tissue has been destroyed; but at least one-half of the organ has escaped destruction at the time of examination, which in most cases is between 20 and 30 days following transplantation. There is, in these cases, a definite lymphocytic infiltration, provided injury to the tissue has not led to a marked diminution in the effectiveness of the individuality differential substances. Grade 1+ (1.25) signifies the survival of only a small part of the thyroid gland. The reaction in the fat tissue is very severe. Grade 1 is applied to results in experiments in rats and guinea pigs in which the thyroid has been entirely destroyed and the fat tissue is largely replaced by fibrous tissue.

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* The work from the Laboratory of Research Pathology of Washington University School of Medicine was carried out previous to June 1, 1941, when this laboratory was discontinued. The experiments mentioned in this paper were done with the aid of a grant from The International Cancer Research Foundation.

The changes in other organs or tissues, on the whole, correspond to those taking place in thyroid and fat tissue and this correspondence makes possible the grading of the results obtained in all tissues from the same donor and transplanted into the same host. For instance, if ovaries are transplanted together with thyroid, cartilage and fat tissue, we find corresponding intensities of reaction and injuries of the various constituent structures of this organ. In the most favorable cases, large follicles, and even corpora lutea, are found. In somewhat less favorable cases only medium-sized or small follicles develop; a still more unfavorable reaction is indicated if merely primordial follicles survive, without undergoing further growth processes. If the reaction is still more severe, no follicles are seen, but merely a cyst and ducts of the germinal epithelium, medullary ducts, spindle-cell connective tissue, and interstitial gland tissue, together with necrotic remnants of the transplanted ovary. At last, only some interstitial gland may be found, or even this tissue may be lacking and necrotic material with fibrous tissue may be all that is left. However, under all circumstances it is necessary to make allowances for the occurrence of accidental injuries to the transplant. This can be recognized in many instances, but even if it should be difficult, errors in the appraisal of the reaction can be avoided by making a series of experiments instead of a single one and then relying on an evaluation of the total results. While this method of grading can claim only approximate exactness, still it is very helpful in comparing the results in the interaction of different types of individuality differentials and in making possible a concise and sufficiently accurate expression of a large number of otherwise separate data.

The principal results obtained in the first series of experiments (1927) are as follows:

SERIES I

Inbred Families A and B

In subseries 1, generations 37 and 38, 40 and 41, in subseries 2, generations 46 and 47 were used.

Subseries 1, family A (different litters): grade 1.96 (20 rats)
Subseries 2, family A (" "): " 1.12 (4 rats)
Total family A (" "): " 1.82 (24 rats)

Subseries 1, family B (different litters): grade 2.1 (22 rats)
Subseries 2, family B (" "): " 1.55 (5 rats)
Total family B (" "): " 1.92 (27 rats)

Families A and B combined: grade 1.87 (51 rats)

Subseries 1, family A to family B: grade 1.74 (11 rats)
Subseries 2, family A to family B: " 1.57 (7 rats)
Total family A to family B: " 1.67 (18 rats)

Subseries 1, family B to family A: " 1.61 (16 rats)
Subseries 2, family B to family A: " 1.33 (3 rats)
Total family B to family A: " 1.57 (19 rats)

Total inter-family A and B transplantations: Combined grade 1.62 (37 rats)

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Exchange of tissues between members of the same litters (brothers and sisters) in families A and B

Subseries 1, family B: grade 2.87 (17 rats) (40th and 41st generations)
Subseries 2, family A: grade 2 (6 rats) (42nd generation)
Total families A and B (40–42 generations): grade 2.5 (23 rats)

Subseries 3, family A: grade 1.37 (6 rats) (46th and 47th generations)
Subseries 4, family B: grade 1.78 (7 rats)
Total families A and B (46–47 generations): grade 1.59 (13 rats)

Total families A and B (members of same litters): grade 2.26 (36 rats)
2 subseries of family A: grade 1.68 (12 rats)
2 subseries of family B: " 2.55 (24 rats)

These data are summarized in Table I.

In evaluating these grades, we must attach greater importance to those experiments in which the figures represent the averages of a large number of rats than to those in which the figures are based on a relatively small number; in the latter, variable factors of an accidental nature may preponderate. This is a point to be considered if there is some discrepancy between the figures in related series of experiments. However, on the whole the figures here agree very well with each other.

The transfer of tissues from members of family A to members of family B, and vice versa, should correspond about to ordinary homoiogenous transplantations. The average grade is, here, 1.62, which is perhaps very slightly better

Table I (Series I)

Donor and host	Grades	Combined grades
Family A to family A (different litters)	1.82 (24 rats)	1.87 (51 rats)
Family B to family B (different litters)	1.92 (27 rats)	
Family A to family A (litter mates)	1.68 (12 rats)	2.26 (36 rats)
Family B to family B (litter mates)	2.55 (24 rats)	
Family A to family B	1.67 (18 rats)	1.62 (37 rats)
Family B to family A	1.57 (19 rats)	

than might be expected. This grade may be compared with that of transplantations between members of family A: 1.82, and between members of family B: 1.92, or an average grade of both kinds of intra-family transplantations: 1.87. There is a slight improvement, a slight diminution in the severity of the reactions in these intra-family transplantations as compared with the inter-family transplantations. Still somewhat higher is the average grade of transplantations between litter mates (brothers and sisters) in family A as well as in family B. Here the total average grade is 2.26 (in the larger series of experiments in family B the average grade is 2.55 and in the smaller series in family A it is 1.68). We may then conclude that as a result of close inbreeding for 37 to 47 generations in families A and B, only a very slight progress towards a homozygous condition has been accomplished.

Series II. In this series of transplantations, rats inbred for 60 to 67 generations were used. The following is a summary of the principal results in this series.

If we compare the grades in the first and second series, we find that transplantations between different litters in family A, and again in family B, show no improvement in series II over the corresponding grades in series I; on the contrary, the average grades in series II happen to be somewhat lower. This may perhaps have been due to a smaller number of brother-sister matings which different litters in series II had in common. On the other hand, in the latter series the grades between litter mates in family A, as well as in family B, are not only better than the grades obtained in transplantations between different litters, but they are also better than the transplantations between litter mates in series I. If the results of transplantations between brothers and sisters are compared in inbred rats, the variable factor of the branching-off of certain lines at different levels of brother-sister matings is eliminated and the improvement in series II in this type of grafts may thus indicate a certain progress towards a homozygous condition, although this cannot be maintained with certainty. Exchange of tissues between families A and B (inter-family transplantations)

TABLE II (Series II)

Donor and host	Grades	Combined grades
Family A to family A (different litters)	1.16 (16 rats)	1.49 (49 rats)
Family B to family B (different litters)	1.65 (33 rats)	
Family A to family A (litter mates)	2.60 (17 rats)	2.71 (36 rats)
Family B to family B (litter mates)	2.81 (19 rats)	
Family A to family B, or family B to family A	1.37 (32 rats)	
Homoiotransplantation in non-inbred families	1.36	
Hybrids $(A \times B)F_4$ (or F_5) to hybrids $(A \times B)F_4$ (different litters)	1.29 (12 rats)	
Hybrids $(A \times B)F_4$ (or F_5) to hybrids $(A \times B)F_4$ (litter mates)	1.80 (26 rats)	
Family A or family B to hybrids $(A \times B)F_4$	1.50 (13 rats)	
Hybrids $(A \times B)F_4$ to family A or family B	1.39 (13 rats)	

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corresponds to a severe homoio-reaction, as might be expected. Similar are the grades of transplantations between hybrids $(A \times B)F_4$. These grades are improved to a certain degree if hybrids which are litter mates are used. But in these transplantations the results are not so good as in brother-to-brother transplantations in family A or in family B. Such a difference might be expected, because in hybrids there is a greater chance for unlike genes to accumulate in brothers when both A and B may contribute to the genes of the fertilized germ cells.

Transplantations from parent to hybrid give somewhat better results than the reciprocal transplantations, but both elicit severe homoiogenous reactions as an indication that a homozygous genetic constitution has not yet been reached in either family A or family B. It is of interest to note that in both series I and II the grades in family B are higher than in family A. On the whole, then, while it is possible that a further slight progress towards a homozygous condition in family A and in family B has been made in continued propagation by brother-sister matings in the interval between the 37th to 47th generations and the 60th to 67th generations, this progress, if present at all, is not very marked.

Series III. This is the series of experiments which has been carried out during the last few years and it will be discussed, therefore, in somewhat more detail. A small number of experiments were made with rats of the 91st and 92nd generations. These were young animals and at the end of the experiments, weighed between 125 and 140 grams. Transplantations into three litter mates, serving as hosts, gave the grade 2.95; very closely approaching the results obtained in autogenous transplantations. In seven transplantations from family B to family A the grade was 1.48, if the examination took place after 20 days, and it was 1.84 in 7 rats after 12 days. The grade after 20 days was very similar to the grades obtained in the corresponding transplantations in series I and II. No essential changes have taken place in this type of grafting between two different families in the course of long-continued brother-sister matings. The grades for transplantations between litter mates are somewhat better than in the corresponding ones in series II, and they are distinctly better than in series I, indicating further progress to a homozygous condition after 91 to 92 continuous brother-sister matings. However, in evaluating the grades obtained in transplantations between litter mates, the small number of experiments, of which this figure represents the average, has to be considered.

In generations intermediate between the 92nd and 104th generations, family B died out and from then on only family A and hybrids $(A \times B)F_4$ or $(B \times A)F_4$ were available for experiments. A number of transplantations were carried out with generations 104 to 106 of family A and with the hybrids. Younger rats, one month and one or two weeks of age, or somewhat older ones, four and a half to five and a half months of age and weighing between 185 and 200 grams at the beginning of the experiments were used for this purpose. Examination as a rule took place after 20 days. Usually three pieces, namely, thyroid, cartilage with fat tissue, and striated muscle tissue were transplanted, and in some experiments the transplants from one kind of donor were transferred to the right side, and those from another kind of donor were transplanted to the other side of the same host.

Group A. Transplantations of organs in young rats

(*a*) Transplantations between members of different litters in family A: average grades, 2.81 and 2.83; (*b*) between litter mates: average grades, 3.15; 3.15; 3.12 and 3.07; (*c*) from hybrids F_4 between families A and B to family A: average grades, 3.12 and 3.10; (*d*) between litter mates of hybrids $(A \times B)F_4$ or $(B \times A)F_4$: average grade, 3.12—the same grade was found between hybrids which belonged to different litters. There is no difference, or only a very slight one, in the reactions obtained in these various transplantations, the grades between non-litter mates being somewhat lower than those between litter mates; but the significance of this difference is not great, because only four transplantations were carried out between members of different litters. On the other hand, even the grades for transplantations from hybrids F_4 to family A, which might have been expected to lead to more severe reactions, were as good as those between litter mates of strain A. We must therefore assume that the young age of these rats helped to diminish the severity of the reactions, which was also diminished by the long-continued inbreeding, causing an approximately autogenous condition of the individuality differentials in family A and in those members of family B which were components of hybrids $(A\times B)F_4$. However, in one experiment the transplantations from hybrids F_4 to family A, in accordance with expectations, gave a somewhat more severe reaction; the average grade was here slightly below 2. In this case, the host reacted definitely against two transplants which contained strange B genes; the threshold which determines an antagonistic reaction had evidently been passed.

Group B. Transplantations in somewhat older rats

These rats varied in age between four and a half and seven and a half months and belonged to the 102nd, 103rd, or 104th generation. The kind of transplantations were about the same as in the younger animals. Transplantations were made (*a*) between litter mates of family A: grade 3.04 in rats aged $4\frac{1}{2}$ to $5\frac{1}{2}$ months, and grade 2.56 in rats aged $6\frac{1}{2}$ to $7\frac{1}{2}$ months; (*b*) between different litters of family A: grade 2.93 (age of rats $4\frac{1}{2}$ to $5\frac{1}{2}$ months), and grade 2.4 (age $6\frac{1}{2}$ to 7 months). The grades between litter mates are only very slightly better than those between members of different litters in family A. These grades are not as good as those in the younger rats, and also among the older rats the severity of the reaction increased somewhat with increasing age; transplantations were also made (*c*) between hybrids $(B\times A)F_4$ and hybrids $(A\times B)F_4$: grade 2.18; (*d*) from family A to hybrids $(A\times B)F_4$: grade 2.39; (*e*) from hybrids $(A\times B)F_4$ or $(B\times A)F_4$ to family A: grade 2.23. The grades in transplantations between hybrids themselves and between hybrids and members of family A, in accordance with expectations, are less good than those between members of family A. These differences come out in the older rats, while they are not present in the corresponding transplantations in younger rats. However, also in the older rats there are no very sharp differences between these various groups.

We shall now give brief abstracts of our microscopic findings in some of these transplantations, which may serve as examples. (1) Transplantations in young rats, family A, inbred for 104 or 105 generations. These rats were 37 to 44 days old at the time of transplantation; examination took place 20 days after transplantation. Pieces from litter mates were transplanted to the right side and pieces from different litters to the left side of the host. There was no definite difference between pieces from the two sides. The transplant of striated muscle tissue was well preserved. There were well-preserved, thin muscle fibers, either close together or separated by some hyaline tissue. Some thick fibers were surrounded by hyaline tissue and in certain instances also by strands of fibroblasts. Necrotic muscle fibers were, in places, surrounded by foreign-body giant cells. There were well-developed nuclear chains, but there was less tendency to the formation of nuclear masses, less crowding, and better cross-striation of muscle fibers in the rat than in the mouse. On the whole, the multiplication of nuclei in fibers was moderate. On the average, the least proliferation of muscle nuclei was found where the thickness of the muscle fibers was greatest and where the cross-striation was best. Where the muscle fibers were very thin and much drawn-out, they often resembled fibroblasts. Some muscle fibers might be changed into a light material, which did not stain with eosin.

The thyroid transplants were well preserved. There was a ring of two to three layers of acini of medium or small size. The acinar epithelium was flat cuboidal, or, occasionally, cuboidal in shape; the center consisted of fibrillarcellular connective tissue, in which there were some lymph and blood vessels. There were also some ducts lined with squamous epithelium, or there were some epithelial pearls in the center of the transplant, and here, too, fat cells were found, indicating a favorable result of the transplantation.

Cartilage and fat tissue were well preserved. The strands of fibrous tissue observed in the fat tissue presumably were caused by the injury sustained during operation. Otherwise there was no connective tissue ingrowth, no infiltration with lymphocytes, and no accumulation of small vacuolated cells between the fat cells. Necrosis occurred in a small piece of cartilage—probably as the result also of injury at the time of transplantation—and surrounding the latter was a plate of regenerated perichondral cartilage; but in the piece from the left side of the host, some necrotic cartilage, without regenerated cartilage plate, was seen.

As to the behavior of the lymphocytes in the muscle tissue from the right side (litter mates), there were merely a few lymph vessels filled with lymphocytes. In the piece from the left side (non-litter mates) there were some similar lymph vessels, and, in addition, some diffuse lymphocytic infiltration. In the thyroid transplant from the right side the lymphocytic infiltration was very moderate; there were a few lymph vessels filled with lymphocytes, and at the two poles, small collections of lymphocytes were seen. In the thyroid transplant from the left side a dense lymphocytic infiltration was noted in the center of the gland around squamous cell nests. In places, lymphocytes penetrated between acini and separated them. Lymph vessels were stuffed with lymphocytes. Also, the parathyroid showed lymphocytic infiltration. In the cartilage-fat tissue transplants of the right side (litter mates) there were no lymphocytes. On the left side (non-litter mates) there was some lymphocytic infiltration in the fat tissue along the living cartilage. This experiment exemplifies well the correspondence between multiple transplants from the same donor into the same host.

(2) Transplantation in young rats from $(B \times A)F_4$ to A (right side), and from $(A \times B)F_4$ to A (left side). In the muscle transplants on the right and left sides there was some lymphocytic infiltration in various places between groups of muscle fibers. On the right side the muscle was less well preserved, more necrosis and more lymphocytic infiltration had taken place, and many nuclear masses were seen. Around some cloudy muscle fibers, foreign-body giant cells had formed. In both thyroid transplants there was intense lymphocytic infiltration; the ring of acini was incomplete; some groups of acini were separated by lymphocytic masses. Lymphocytes penetrated also into the colloid, where they disintegrated. The masses of lymphocytes had destroyed a number of acini. In the cartilage-fat tissue, cartilage was well preserved. On the left side there was more fat tissue replaced by fibrous tissue than on the right side. In both transplants there was some lymphocytic infiltration along the perichondrium and, in some places, also between the fat cells. In these experiments the reaction is more severe than in transplants from A to A.

(3) In the somewhat older rats, the grades in some of the transplantations within the inbred family A approached those of autogenous reactions. There were occasionally slight lymphocytic infiltrations in the thyroid, in which there were several layers of acini, but there were no lymphocytes between the acini. The muscle transplant was well preserved, with much cross-striation and with nuclear chains and masses. The fibers with medium thickness were best pre-

served as a rule. Some muscle fibers became hyaline and the preserved fibers were usually embedded in hyaline tissue. There were occasionally some small collections of lymphocytes in the transplant. In the cartilage-fat-tissue transplant some thickened septa with a small number of lymphocytes were observed.

(4) $(B\times A)F_4$ to $(A\times B)F_4$ (older rats—experiment 8). The muscle transplant showed very intense lymphocytic infiltration throughout, and this destroyed the fibers. Certain muscle fibers disintegrated, others became hyaline. There were some nuclear chains. The thyroid ring was incomplete. Single acini might be separated by connective tissue and lymphocytes. Much lymphocytic infiltration had taken place and lymph vessels might be stuffed with lymphocytes. The lymphocytes penetrated between the acinar cells into the colloid. The greater part of the thyroid had been destroyed. In the cartilage-fat-tissue transplant some cartilage had become necrotic and dense fibrous tissue surrounded it. Much fat tissue was destroyed, but there were still some groups of fat cells. In some places more fat tissue was preserved, with fibrous strands and definite lymphocytic infiltration in the hyaline connective tissue. Lymph vessels were stuffed with lymphocytes in the connective tissue and fat tissue.

(5) Experiment 61b. 91st and 92nd generations, family B to family A; (20 days). In the thyroid the results varied between those in which no thyroid was left, to those in which the remnant of the transplant consisted mainly of compressed acini, with much lymphocytic infiltration and some connective tissue ingrowth. In the cartilage-fat-tissue transplant, much fat tissue, and in some places almost all of it, was replaced by fibrous tissue. If much lymphocytic infiltration occurred in the thyroid, usually a certain amount was found also in the fat tissue. Bone marrow was not preserved, but as a rule some transplanted muscle fibers were preserved. Some lymphocytic infiltration had taken place in this transplant.

The principal results obtained in series III are summarized in Table III.

Table III shows that the grades in the younger group of rats are consistently higher than in the somewhat older rats; this consistency in the difference between the grades in these two groups makes it very probable that this difference is significant, even in cases in which the averages are based on a small number of individuals. The differences are, however, not very great in transplantations within the inbred family A; they are greater if we compare the transplantations between hybrids and from hybrids to parent-family A in the old and young groups. The grades in transplantations from family B to family A, both belonging to the 91st and 92nd generations, are very low; as was to be expected, they correspond to homoiogenous reactions.

If we compare the grades obtained in series III with those of series II, we notice that they are much better in the former. In transplantations within family A, the grades have advanced from 1.16 in series II to about 2.75 in series III. Among litter mates within family A they have advanced from 2.60 in series II to about 2.90 in series III. This improvement is not so great as in the transplantations between non-litter mates, which is due to the fact that there was already a considerable improvement among the grafts between litter mates in series II, as compared to those in series I with a corresponding grade of 1.68. There has been also a great improvement in the grade of transplantations between hybrids F_4 (non-litter mates). The corresponding grades are 1.29 in the II

TABLE III (Series III)

Donor and host	Grades	Grades
A to A (different litters)	2.82 (7 rats)	2.72 (10 rats)
A to A (litter mates)	3.10 (22 rats, including 3 from the 91st and 92nd generations)	2.77 (7 rats)
$(A \times B)F_4$ and $(B \times A)F_4$ (different litters as hosts and donors in various combinations)	3.12 (6 rats)	2.18 (2 rats)
$(A \times B)F_4$ to $(A \times B)F_4$ (litter mates) $(B \times A)F_4$ to $(B \times A)F_4$	3.12 (6 rats)	
A to $(A \times B)F_4$		2.39 (4 rats)
$(A \times B)F_4$ or $(B \times A)F_4$ to A	2.87 (10 rats)	2.23 (4 rats)
B to A (91–92 generations)	1.48 (7 rats) 20 days 1.84 (7 rats) 12 days	

series and about 2.50 in series III. Transplantations from hybrids to family A (or B) show a considerable improvement from grade 1.39 in series II to about grade 2.50 in series III; and between litter mates of hybrids there is a change from 1.80 (series II) to 3.12 (young rats) in series III. In transplantations from family A to hybrids the advance is marked, in series II the grade being 1.50, in series III, 2.39 (older rats). Where in series III there were grades available in both groups, namely, in those of old and of young rats, a somewhat arbitrary intermediate grade was chosen, but one nearer to the grade of the older rats. In contrast to the change which has taken place in the interaction between the individuality differentials of the members of inbred strains, in the course of long-continued brother-sister matings, no essential change has occurred if the individuality differentials interacted between members of family A and of family B. In this case, the grade (1.48) obtained in series III, when the examination took place after 20 days, indicates a typical homoiogenous reaction. As was to be expected, the reaction was not yet fully developed in its full strength and the grade therefore was somewhat higher (1.84) if the examination took place after 12 days.

DISCUSSION

A comparison of the reactions observed in these three series of transplantations shows that a continuous progress to a homozygous condition has been made. In the first series, there was only a slight indication of an improvement in grades over the grades in ordinary homoiogenous and syngenesious transplantations. A further slight progress was noted in the II series, but the greatest advance was made in the interval between the II and III series. The first series comprised the 37th to 41st generations; the second series, the 60th to 67th generations; and the III series, the 91st and 92nd generations as well as the 102nd to 107th generations. After about forty generations, there was then only a very slight progress towards an autogenous character of the individuality differentials; some advance was made after 60 to 67 consecutive brother-sister transplantations, and still more in the 102nd generation; but even at that time, no completely homozygous condition had been attained. This finding is indicated especially by the transplantations into which the hybrids entered; but it is noticeable also in the transplantations within the inbred family A. Of interest in the third series is also the difference in the grades in the group of the young and the somewhat older rats, which agrees with the general observation that when donors and hosts are very young, the reactions are milder than in older animals. This difference cannot be due to a lack of individuality differentials in the former, because they are present; but it is due, rather, to a lesser sensitiveness to strange individuality differentials or to a not yet fully developed mode of reaction in the younger animals. In addition, the fact must be taken into account that younger tissues grow more vigorously than older ones, and this condition is associated with a greater ability to overcome the effect of the antagonistic reactions of the host; it may also be that tissues growing more rapidly do not give off individuality differential substances in as large amounts as the more differentiated tissues, which metabolize in carrying on their function. In accordance with these considerations, we noticed that in the group of younger rats the grades are higher even in transplantations from hybrids to an inbred parent strain, where the derivatives of strange genes are introduced into the host. Another important conclusion to be drawn from these experiments is that although so large a number of consecutive brother-sister transplantations were carried out, a completely homozygous condition has not yet been reached in these families, as is shown by the results of transplantations within the inbred family. This conclusion agrees with the findings obtained also in transplantations in inbred families of guinea pigs and mice. In none of these, even after years of consecutive brother-sister matings, has a completely autogenous state of the individuality differentials been attained. This effect, which is contrary to expectation if merely the distribution of chromosomes during fertilization is considered, is presumably due to the occurrence of random mutations in the germ cells, which prevent a complete homogeneity between the various individuals of an inbred strain. However, these mutations are able merely to delay but not entirely to prevent an approach to a completely homozygous condition, although this desired endstage, on account of such mutations, may never be fully reached by close inbreeding. There was, however, a difference in the rapidity with which progress in the direction towards this endstage was accomplished; it was more rapid in inbred guinea pigs than in inbred rats. In rats, a selection was made of the strongest members of each litter for the purpose of propagation. Such a selection was not made in the case of the guinea pigs. But, it is not probable that this factor would have been so influential as to cause the difference between the results of inbreeding in these two species. Such a difference may possibly be due to differences in the rate of mutations in guinea pig and rat. There is no indication that the sensitiveness of the host for and his reactivity against homoiogenous individuality differentials is greater in the rat than in the guinea pig.

Conclusion

In the course of more than one hundred consecutive brother-sister matings in family A, and of somewhat less than one hundred brother-sister matings in family B of King's inbred albino rats, a gradual increase in the homozygous condition of the inbred families has taken place, as indicated by the lessened severity of the reactions of the hosts against the individuality differentials of various members of these families. However, the advance was slight in the course of the first 67 generations of brother-sister matings; it was greater between the 67th and the 102nd generations; but even at the latter point a completely homozygous condition in the inbred family A had not yet been attained. These results were obtained in testing the interaction of the individuality differentials in litter mates, in members of different litters, as well as in hybrids of these inbred strains.

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FACTORS INFLUENCING GROWTH AND METAMORPHOSIS OF THE SALIVARY GLAND IN DROSOPHILA

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Introduction

The present investigations are concerned with the development of the salivary glands in Drosophila. The larval salivary glands are strictly larval organs and grow by increase in cell size. They are completely histolysed during the early part of pupal life. The salivary glands of the adult fly, on the other hand, are imaginal organs which develop from imaginal discs situated at the extreme proximal end of the larval salivary gland, and grow by cell multiplication. Thus both larval and imaginal salivary glands undergo a period of growth during larval life, while metamorphosis leads to the differentiation of the imaginal and the destruction of the larval salivary glands. From the work of Hadorn (1937) we know that the principle causing pupation in Drosophila is a hormone released by the ring gland, a small glandular organ situated dorsally between the two hemispheres of the larval brain. The role of the ring gland in the further differentiation of the various organs involved in the process of metamorphosis is, however, still obscure. Furthermore, virtually nothing is known about the causal factors concerned in the growth of organs during larval life. The present study attempts to analyze some of the causes which underlie the visible expression of growth and differentiation in the development of the salivary glands.

Material and Methods

Drosophila virilis was used for this investigation. Experimental animals were kept at a constant temperature of 25° ± 0.5° C. Instead of the usual method of transplanting larval tissues into larvae, the body of the adult fly was used as a carrier of the larval transplant. The larval tissues transplanted into the body cavity of the adult fly live in their new environment for a long time, perhaps indefinitely. They do not lose their developmental potencies; they grow and differentiate normally when provided with the appropriate stimulus. The adult hosts withstand the operation, which is simple in method, very well. The mortality rate is negligible even when the same host is used for continued transplantations. Indeed this new method approximates tissue culture more closely than any other so far known for insects. Its great advantage lies in the fact that one is able to study the developmental behavior of larval tissues outside their own larval environment. All histological observations on the salivary gland are based on orcein-stained material which was examined either in total mounts or in smear preparations. For certain developmental characteristics the glands were also examined in the living condition.

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It gives me great pleasure to extend my sincerest thanks to Drs. L. C. Dunn, Th. Dobzhansky, F. Schrader and S. Hughes-Schrader for many stimulating discussions concerning this work and for their continued interest in the progress of these investigations. I also wish to express my appreciation of the assistance given by my wife.

NORMAL DEVELOPMENT OF THE SALIVARY GLAND

In the normal development of the salivary gland one is confronted with a sequence of developmental steps, each of which is characteristic for a definite stage of development. Since the normal development of the salivary gland is necessary for the understanding of the experimental part of this paper, it will be discussed briefly. However, only such features of development will be set forth as are of importance for our special problem.

The salivary glands are paired organs. They develop as invaginations on either side of the anterior ectoderm. As development proceeds these invaginations elongate, grow inward, and unite medially into a common duct leading to the pharyngeal cavity. (Sonnenblick, 1940.) When the embryo hatches, the number of cells in each gland is, according to Makino (1938), about 115 and it does not change during larval life. The growth of the larval gland is, as already mentioned, the result of increase in cell size rather than cell multiplication. The development of the salivary gland has been staged from the time the larva leaves the egg to the time the gland is histolysed. Eleven successive stages of development are shown in Plate I, which represent photomicrographs of salivary gland total mounts, stained with orcein. In Plate II the distal portion of the same glands is shown at a higher magnification. Camera lucida drawings at a magnification of 85× were made for each gland of this normal series, as well as for the glands used in the experiments, and their circumference measured with a planimeter. The size values thus obtained together with special histological landmarks characteristic for the different developmental stages, assured great accuracy in the determination of the various stages. In the following the measurements of the circumferences are given for the normal series of development.

stage of development	1	2	1st molt	3	4	2nd molt	5	6	7	8	9	10–11
circumference (× 85) in cms.	2.5	4.0	6.0	9.0	11.0	12.5	14.0	17.0	23.0	28.0	41.0	meta.

The histological changes that occur during the development of the salivary gland are now set forth. The observations are based on orcein-stained total mounts.

Stage 1 (Plate I, Figure 1 and Plate II, Figure 1′).

The cell size is uniform throughout the gland. The cells are very small and the cell borders not clear. No details are visible in the nucleus.

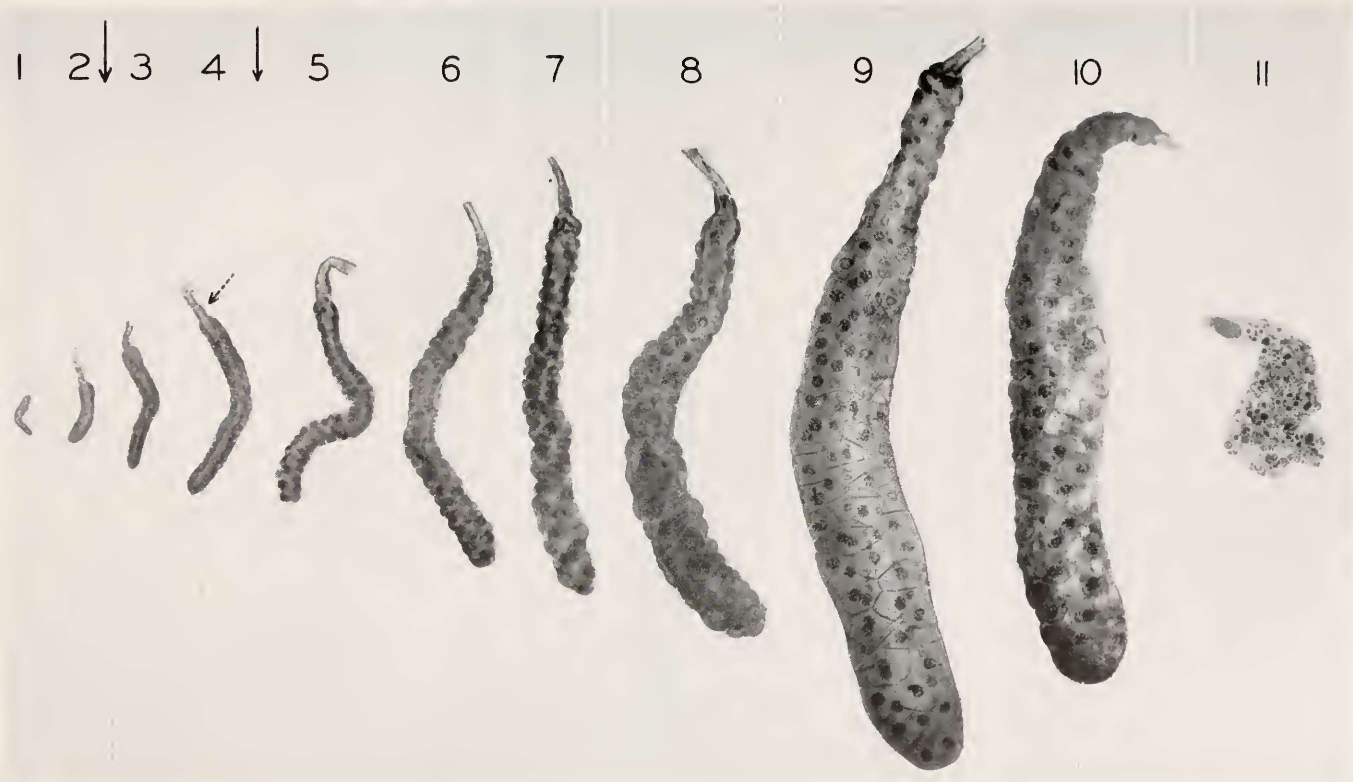


Plate I. Normal developmental series of the larval salivary gland of Drosophila virilis. Figures 1 to 11: Photomicrographs of 11 successive stages of development. (Orcein stained total mounts.) Full arrows indicate time of molting. Broken arrow shows position of imaginal salivary gland anlage. (For explanation see text.)

Stage 2 (Plate I, Figure 2 and Plate II, Figure 2′).

The cell size is uniform throughout the gland. The chromatin forms a fine net-work around the edge of a clear area in the nucleus. The chromocenter is visible in all cells and stains deeply.

First molt. The cells are larger than in stage 2; otherwise there is no change.

Stage 3 (Plate I, Figure 3 and Plate II, Figure 3′).

The cells are larger; otherwise unchanged.

Stage 4 (Plate I, Figure 4 and Plate II, Figure 4′).

The cells are larger; they are still uniform in size throughout the gland. The chromatin forms a net-work consisting of fine strands around the edge of a clear area in the nucleus. The chromocenter is visible in all cells and stains deeply. At the proximal tip of the gland, where it borders the duct, a small number of tiny cells becomes visible; these represent the imaginal anlage of the adult salivary gland. (See Plate I, Figure 4, arrow.)

Second molt. The cells in the distal portion of the gland have become larger in size than those of the proximal portion. The chromosome strands, especially those in the nuclei of the distal gland portion, have become wider; alternating deeply stained areas and lightly stained areas are clearly distinguishable within the individual strands. The deeply staining chromocenter is still visible in all cells. The imaginal anlage of the adult gland has become very clear and its cells form a ring-like structure around the salivary duct.

Stage 5 (Plate I, Figure 5 and Plate II, Figure 5′).

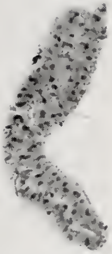
The cells in the proximal gland portion are much smaller than in the distal; this difference is very pronounced. The chromosome strands have become wider and the chromatin bands more distinct. The chromosome strands on the periphery of the nucleus embrace the clear area in the center of the nucleus like octopus arms. The chromocenter is still visible in all cells. The imaginal ring cells have increased in number.

Stage 6 (Plate I, Figure 6).

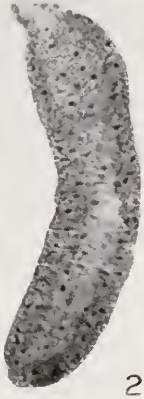
The cells are larger; otherwise unchanged.

Stage 7 (Plate I, Figure 7 and Plate II, Figure 7′).

The difference in cell size between the proximal and distal gland portions is very clear. The chromosome strands are broad and the chromatin bands very distinct. The chromocenter has become invisible in the cells of about one-half of the distal portion of the gland. To avoid misunderstanding this point has to be made clear. The staining capacity of the chromocenter at this stage has by no means changed considerably, as smear preparations show. It has become invisible in total mounts because the chromosome strands in the distal cells have grown so much in width as to eliminate the former discrepancy in width between chromocenter and strands. Therefore if we speak in the following of the disappearance of the chromosome center, we mean just this fact. The disappearance

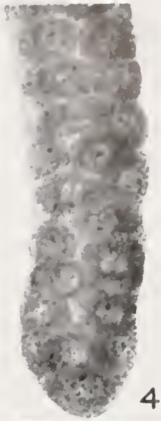


1′

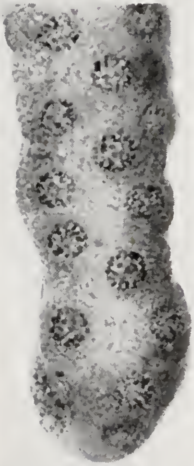


2′

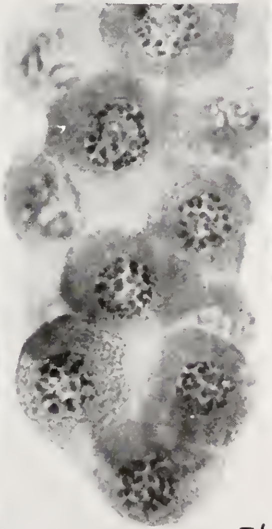
3′



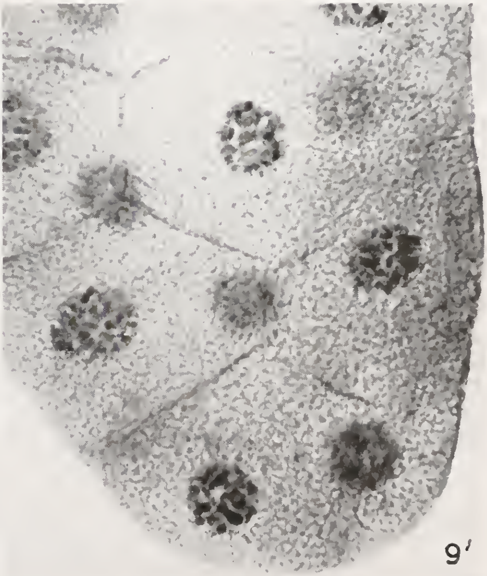
4′



5′



7′



9′

Plate II. Figures 1′ to 9′: the distal portion of the same glands as shown in Plate I, Figure, 1 at a higher magnification. (For explanation see text.)

of the chromocenter is hence a good indication of the nuclear size. The imaginal ring cells have increased in number.

Stage 8 (Plate I, Figure 8).

The difference between the cell size in the proximal and distal part of the gland is very pronounced. The proximal cells have reached a size which corresponds approximately to the cell size in the distal part of the preceding stage. At no time, however, do we find any sharp separation into a proximal and distal portion of the gland as far as cell size is concerned. On the contrary, the cells increase gradually in size in a proximal-distal direction. Because of this differential growth, the salivary gland acquires its characteristic shape. The form and size of the salivary gland seem thus to be determined by two different growth rates in two different directions; one correlated with age affects the gland as a whole, and the other constitutes a proximal-distal gradient which determines the size of the cells throughout the length of the gland. While up to this time cell growth took place by an increase in size of the nucleus and cytoplasm simultaneously, at this stage a remarkable change occurs in the cells of the distal half of the gland. The nuclei in these large distal cells cease to grow, or grow only very little indeed, yet the cytoplasm increases immensely. A comparison of (Plate II) Figure 7′ with 9′ illustrates this point quite clearly. The chromocenter has become invisible in the distal two-thirds of the gland and the chromatin bands in the chromosome strands especially of the distal cells stain very distinctly. The imaginal ring has increased in size.

Stage 9 (Plate I, Figure 9 and Plate II, Figure 9′).

The larvae are full grown, they have left the food and are ready to pupate. In the living condition the cytoplasm of the glands has become slightly opaque instead of being transparent as in previous stages; this condition is the first indication of metamorphosis. The opaqueness is usually somewhat more pronounced distally. The proximal cells are still smaller than the distal ones. The cells in the distal two-thirds of the gland are extremely large, due to an increase in their cytoplasm, while their nuclei are of about the same size as in stage 8. Although the nuclei are unaltered in size, their condition has changed. The clear area in the center of the nucleus has disappeared. The chromosome strands have become more compact, winding their way through the whole nucleus. The individual strands show their characteristic banding most perfectly. The cytoplasm stains less heavily than in previous stages, while the nuclei take the stain exceedingly well. One of the characteristic features of this stage is that in total mounts the nuclei stand out clearly against a lightly stained background (Figures 9 and 9′). The chromocenters are visible only in the proximal one-fifth portion of the gland. The imaginal ring has become larger; the chromosome strands stretch very well in smear preparations, in contrast to earlier or later stages. This stage is hence best suited for studies on the arrangement of bands in the chromosome strands.

Stage 10 (Plate I, Figure 10).

Histolysis of the glands begins about 10 hours after puparium formation. In various regions, usually first in the distal part of the gland, the cells begin to vacuolate and the cell walls rupture, while the nuclei are still intact. The cytoplasm in these regions of degeneration stain poorly. The chromosome strands on the other hand stain very deeply. They are clumped in the center of the nucleus and are surrounded by a clear spherical area (Figure 10). In living conditions the glands appear much more opaque, while milky-white zones indicate the regions of advanced histolysis. The chromocenter is by now visible only in a few cells at the extreme proximal end of the gland. The imaginal ring has increased in size.

Stage 10–11 (Plate I, Figure 11).

The next stages of histolysis proceed very rapidly. The degenerating regions within the gland which stain poorly, and which in life appear milky, extend and become more frequent. The nuclei become picnotic. The basement membrane which surrounds the gland breaks down. Finally, about 25 hours after puparium formation, with the probable help of phagocytosis, the larval gland is dissolved. The proximal part is the last to disappear. The differentiation of the imaginal ring begins and is completed during the rest of the pupal period, leading to the formation of the adult salivary gland.

Experiments

The induction of premature metamorphosis.

The description of normal development has revealed that the metamorphosis of the salivary gland consists of the histolysis of the larval and the differentiation of the imaginal salivary gland. The question now arises whether the characteristic developmental behavior of the gland during metamorphosis is dependent on or independent of the conditions prevailing in the animal during metamorphosis. This can be tested by transplanting young salivary glands into older hosts, thus exposing the gland prematurely to the metamorphosis factors.

Salivary glands of stage 5 were transplanted into the abdomen of older larvae shortly before pupation. The transplanted glands were dissected and their condition studied after they had remained in the hosts for various lengths of time. The results of the experiments are summarized in Table I A. The first animals in this series were dissected two days after the operation, when the hosts were about one-day old pupae. The transplanted glands had reached stage 10, i.e. they showed clear signs of histolysis. Their state of development corresponded closely to that of the normal host gland. Yet if the transplanted glands had been left in their own environment, they would have developed certainly not further than stage 8 by this time. Thus, under the influence of the metamorphosis factors of the host, the grafted glands have metamorphosed prematurely. The transplanted glands reached an advanced stage of histolysis when they were left three days in the host, and they disappeared completely when left in the host for six days or longer.

In these transplantations the anlage of the imaginal ring was included in the larval graft. Now it was found (Table I A), that the imaginal gland, like the larval gland, is able to metamorphose precociously when exposed to the metamorphosis factors prematurely. This becomes evident from the observation that the transplanted imaginal anlage in the newly emerged host, i.e. six to seven days after the operation, is already completely differentiated into the imaginal salivary gland (Plate III, Figure 4), while donor controls by this time would have been two days old pupae with quite undifferentiated imaginal glands.

In summarizing the experiments at this point, we find that the metamorphosis of purely larval structures, as well as imaginal structures, is not autonomous, but is caused by some factors in the organic environment of the host during the period of metamorphosis.

Table I A

Transplantation of young salivary glands into larvae shortly before pupation.

A. Transplantations of larval glands of stage 5.

Circumference of transplanted gland	Days transplant remains in host	Host stage dissected:	Stage of transplanted larval gland	Stage of transplanted adult gland
14.0	2	pupa	stage 10+	larval
15.0	2	pupa	stage 10−	larval
15.0	2	pupa	stage 10+	larval
15.5	2	pupa	stage 10−	larval
15.5	2	pupa	stage 10−	larval
13.5	3	pupa	stage 11−	larval
13.5	3	pupa	stage 11−	larval
15.5	3	pupa	stage 11+	larval
15.5	3	pupa	stage 11−	larval
15.5	3	pupa	stage 11−	larval
13.5	6	adult	completely histolysed	adult gland formed
13.5	7	adult	completely histolysed	adult gland formed
14.0	7	adult	completely histolysed	adult gland formed
13.5	8	adult	completely histolysed	adult gland formed
13.5	10	adult	completely histolysed	adult gland formed
15.5	13	adult	completely histolysed	adult gland formed

Table I B

B. Transplantations of larval glands of stage 3.

7.5	2	pupa	stage 6	larval
7.5	3	pupa	stage 10−	larval
7.5	3	pupa	stage 10−	larval
9.0	3	pupa	stage 11	larval
7.5	6	adult	stage 11−	larval
7.5	6	adult	stage 11−	larval
7.5	6	adult	stage 11	larval
7.5	7	adult	stage 11−	larval
9.0	9	adult	stage 11	larval
9.0	9	adult	stage 11	larval
9.0	9	adult	stage 11	larval
9.0	9	adult	stage 11	larval
7.5	10	adult	stage 11	larval
7.5	15	adult	stage 11	larval

The next question to be answered is whether salivary glands younger than stage 5 are competent to respond to the metamorphosis factors. For this, salivary glands of stage 3 were transplanted into older larvae shortly before pupation. The region of the imaginal discs was again included in the graft, but in contrast to the previous series of experiments the imaginal ring as such was not yet morphologically visible. The results of these experiments summarized in Table I B show that histolysis of the young transplanted glands begins about two days after the operation, but is never completed, although the glands may remain as long as 15 days in the host. Young glands which have remained for three days in the host only begin their histolysis, while older glands are at this time quite extensively histolysed (Cff. Table I A with I B). Thus the young glands are presumably unable to respond at a time when the metamorphosis factors are most effective. We must assume that the metamorphosis factors have become less efficient when the glands have finally reached their responsive stage, and are hence unable to induce complete metamorphosis, because it is difficult to understand why the glands should not be histolysed completely if the metamorphosis factors were still active in the late pupal or adult stage.

The inability of the young organ to respond to the metamorphosis factor is demonstrated in the behavior of the adult gland (Table I B). In spite of the fact that the young imaginal gland discs have remained in some cases for a considerable length of time in the host, they show no signs of metamorphosis (Plate III, Figure 3). Although the anlagen have developed well beyond their stage of transplantation and have acquired their typical ring-shaped form, they remain larval and never surpass this stage. Their state of development corresponds to stages 10 and 11 of the normal developmental series, yet this stage, as previous experiments have shown, by far exceeds the stage at which the adult gland anlage is able to react. These observations lead necessarily to the conclusion that the larval development of the disc is not interrupted in its new environment, but that by the time the disc has reached its reactive stage the factors necessary for promoting metamorphosis are absent or not effective enough. The specific results of this experimental series may then be briefly summarized as follows: the larval glands, as well as the analgen of the adult glands, must reach a certain developmental stage before they are able to respond to the metamorphosis factors. If they reach this stage after the active period of metamorphosis they are unable to metamorphose and persist as larval structures in an imaginal environment (Plate III, Figure 3).

In examining prematurely-metamorphosed young salivary glands, one has the impression that the nuclei in these glands are histolysed before they have attained their fully normal size. The same observation was made in prematurely-metamorphosed young salivary glands which were grown in the body of adult hosts. It is difficult to decide whether this impression is real, since the reduction of the nuclear size is only slight and probably within the limit of normal variation. If true, however, it would mean that premature metamorphosis causes an early cessation of nuclear growth, which is not at all unlikely. In the light of these considerations, it seemed interesting to investigate whether older glands transplanted into younger hosts would grow larger than normally, because under these experimental conditions they would be exposed to the metamorphosis factor later than normally. Salivary glands of stage 7 were therefore transplanted into younger host larvae, the salivary glands of which were at stage 4 at the time of the operation. In other words, salivary glands of the last larval instar were transplanted into hosts of the second larval instar. This experimental series consisted of six hosts which were dissected two days after the operation when the transplanted and host salivary glands were compared. It was found that the host salivary glands had developed to stage 8−. They were still transparent, and showed no signs of metamorphosis. The transplanted glands on the other hand were found to be at stage 9+ and had begun to metamorphose, as indicated by their intensely opaque appearance. Moreover, the chromosome strands of the transplanted gland were distinctly wider than those of the host glands. Yet the strands were definitely not larger than normally, i.e. their size was characteristic for a normal salivary gland of stage 9+. Thus the original question whether it is possible to obtain salivary glands larger than normal has been answered by this experiment in a negative way. Some further conclusions which might be drawn here will be discussed on page 30 in a somewhat different connection.

The effect of the ring gland on the development of the salivary gland.

Until now we have dealt almost entirely with specific reactions of the salivary gland. So far nothing has been said about the factors which govern the processes of growth of the gland during larval life and those of metamorphosis during pupation. In the light of our knowledge of hormone-controlled insect metamorphosis, we expect these factors to be hormonal in nature. The search for the activating principle resolves itself into the problem of locating an organ or organs of internal secretion and demonstrating their action on the developing organ system. There are obviously two alternatives in attacking this problem experimentally. One may attempt to remove systematically various organs of supposedly endocrine nature from the larvae and thus hope to locate the responsible organ by testing the developmental behavior of the operated animal. The difficulty involved in a study of this kind is mainly a technical one, for it is very difficult indeed and at the present time seemingly impossible to perform such an operation in the larvae of Drosophila. The other possible approach open to the investigator is to dissect various organs from a donor larva which can be sacrificed and transplant them to a second larva and then test their effect on the development of the host. Although technically quite feasible, this procedure has one great disadvantage. The effect of the transplanted gland on the host may be counteracted or blurred to a great extent by the hormone supply of the host itself, and thus not detectable. The introduced hormone, moreover, may upset the host system to such an extent that the situation, instead of being clarified, is rather obscured. Apart from these difficulties there is the additional one that both approaches are rather indirect, since the experiments are designed

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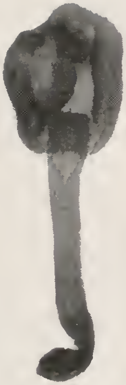
PLATE III, FIGURE 3. Adult salivary gland, developed from the imaginal salivary gland anlage which was transplanted at stage 6 into a host larva shortly before pupation. Transplant dissected from the emerged host six days after the operation.

FIGURE 4. Larval salivary gland persisting as larval structure in the adult host; obtained by transplanting salivary glands at stage 3 into the abdomen of host larvae shortly before pupation. Note: undifferentiated imaginal ring cells (arrows).

FIGURE 5. Paired salivary gland transplantation. Glands transplanted at stage 6 into adult hosts and dissected two days after the operation. *a*. Partner transplanted alone: note bloated appearance of gland and the small nuclear size. *b*. Other partner transplanted together with two ring glands. Note the large, very well stained nuclei with chromosome strands.



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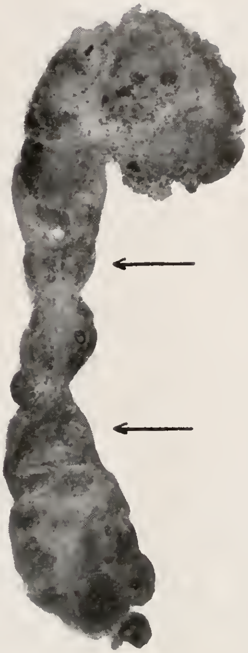

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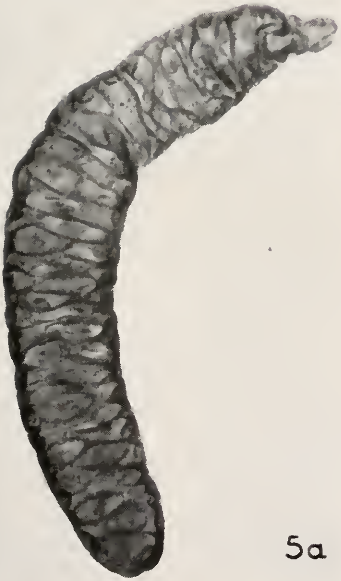

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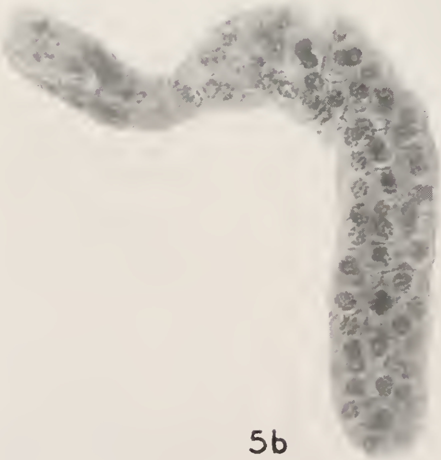

5b

PLATE III

for testing the hormone action on the whole organism rather than on the special organ. We have hence to seek a method by which the hormone action may be more directly studied.

An effort in this direction was made by using the abdominal cavity of the adult male fly as a place for culturing larval tissue in vitro. This environment is supposedly neutral as far as the progress of development is concerned, for the larval salivary glands cease to grow after the transplantation, but continue to live indefinitely. The transplanted tissues do not suffer from a lack of nutrition nor are they unable to utilize the nutritive components of the new environment, as is shown by the following experiment. When young Drosophila larvae are starved, they use the fat stored in their fat bodies and, if starvation is continued, they exhaust their food reserves in the fat bodies completely. Such exhausted fat bodies are strikingly different from the fat bodies of feeding larvae. Now it was found that starved exhausted larval fat bodies restore their food reserves again and become indistinguishable from fat bodies of normally fed larvae when transplanted into the body cavity of adult flies. For these experiments a small strip of fat body closely attached to the salivary gland was transplanted together with the gland. The gland, important in this instance only as a marker, enables one to find and distinguish the transplanted fat body from the fat bodies of the host. The inability of the larval organs to develop in the adult fly is hence not caused by a lack of nutrition but obviously by the lack of some other factor.

Before considering the nature of this developmental factor, one further point has to be cleared up. It has to be shown whether the inability of the gland to develop is typical for glands of all ages or only characteristic for glands of a particular stage of development. In order to elucidate this point, salivary glands of stages 2 to 6 were transplanted into the abdomen of adult male flies. The grafted organs were allowed to remain for various lengths of time in the host before they were dissected out and their developmental condition examined. The results of these experiments summarized in Table II show clearly the inability of the gland to develop in the body of the adult fly regardless of their age at the time of the transplantation and of the length of time they remained in the host. The only perceptible way in which the transplanted glands seemed to be changed while in the adult host was that they became greatly inflated, this being caused by the accumulation of a clear watery fluid in the lumen of the gland. The longer the glands remained in the host, the more fluid accumulated and the more bloated the glands became. The accumulated fluid presumably represents saliva, which is secreted by the gland cells into the lumen of the gland and is unable to escape, since the outlet is closed off in dissecting the gland from the donor larvae. Incidentally, should this fluid really prove to be saliva, we have here a method of accumulating it in order to study its chemical properties.

The foregoing experiments have shown that the larval salivary glands depend for their development upon some factor missing in the body cavity of the adult fly. This factor was found to be the larval ring gland, which when transplanted simultaneously with the salivary glands into the abdominal cavity of the adult fly, caused the latter to continue their development leading to histolysis. The progress of development of the salivary glands was indicated by changes in the nuclei, cell growth and certain characteristic staining reactions. All the typical developmental stages found and described in the normal developmental series may be observed. but they proceed at a much lower rate. The salivary glands are also able to metamorphose, i.e. they become gradually histolysed if left long enough in the adult host. The ring glands used for these experiments came from donor larvae which were ready to pupate. The number of ring glands implanted into one host varied from one to four. Salivary glands of stage 2 to stage 6 were tested for their reaction to the ring gland factor. The transplanted salivary glands remained in the host for various lengths of time. These experiments, summarized in Table II, led to the discovery of a number of essential facts concerning the development of the salivary glands. Glands of all ages tested respond to the ring gland factor. The older the gland is at the time of transplantation, the earlier metamorphosis occurs. This implies that at least younger glands do not metamorphose right away, but grow to a certain point before metamorphosis begins. In the study of normal development we have seen that the first signs of metamorphosis are noticeable in glands of stage 9, as indicated by a slight but distinct opaqueness of the gland in living condition. At this time the chromocenter has become invisible in the distal portion of the gland. We find in glands which develop in adult hosts some variability in the relationship between the onset of opaqueness, i.e. metamorphosis, and the disappearance of the chromocenter. The degree of opaqueness observed in the transplanted glands is indicated in Table II by the Roman numerals I to III placed next to the number which designates the stage of development. The number I represents an opaque condition of stage 9 and the number III of stage 11− in normal development. When no Roman numerals are given, the relation between metamorphosis and developmental stage is normal. The transplanted glands become opaque at an earlier developmental stage. This is especially true in the transplantation of younger glands. For instance, glands which have developed in the adult host to stage 7− show an opaque condition which corresponds to stage 9 or 10 in normal development. Since we know from normal development that the chromocenter becomes invisible because of the thickening of the chromatin threads, it follows that the glands begin their metamorphosis before the strands have developed to their normal size. It was also noticed in many cases that the nuclear size in advanced prematurely metamorphosed glands seemed smaller than normal. This would indicate that they degenerated before they had reached their full size. These facts are in agreement with the experiments described on page 19 where young glands in old larval hosts were made to metamorphose precociously. The number of ring glands transplanted seems to be of no great importance for the development of the salivary gland, since one or three ring glands produce about the same effect.

In all these experiments where adult flies were used as hosts the imaginal salivary anlage usually transplanted together with the larval gland remained larval. Its cell number, however, increased considerably when transplanted together with ring glands. The amount of increase depended upon the time the anlage remained in the host. However, no sign of differentiation could be noticed, in spite of the fact that in some cases more than four ring glands were present and the disc remained for a considerable length of time in the host. In the effort to obtain a more precise comparison between the developmental behavior of larval salivary glands with and without ring glands, the two glands of one donor were compared with each other. For this experiment the paired gland of a single donor was dissected, the two glands separated and one partner transplanted into one adult host without ring glands and the other partner transplanted into a second adult host together with two to four ring glands.

TABLE II

Transplantation of salivary glands of various ages; alone and together with different numbers of ring glands into the abdomen of adult male flies. Roman numerals indicate state of opaqueness of the gland. (For explanation see text.)

Stage of transplanted salivary gland	Days transplant remains in host	Number of ring glands transplanted									
		None		One		Two		Three		Four	
		Number of cases	Stage of development	Number of cases	Stage of development	Number of cases	Stage of development	Number of cases	Stage of development	Number of cases	Stage of development
2 ↓ 2	2	1	3−	1	I 9						
	3			2	5						
	4	2	3	2	I 7−			1	5		
	5									1	4+
	7	2	3−					2	6	1	I 7−
	14	1	3−					1	II 9+		
3	6	1	3			1	I 7+				
	9	2	3			2	II 8				
	13	1	3			1	III 10				
4 ↓ 4	1	1	4					1	5+		
	4	4	3+	1	I 7			1	I 8−		
	6	4	4−	2	I 7					3	10
	7	4	4−					2	II 8+		
	8	3	4+								
	9	2	4+							1	II 8
5	2	1	5−					3	6		
	2	1	5					2	II 9		
	5	1	5							1	I 7
	6	1	5			1	8				
	7							1	II 9−		
	9	2	5			2	10+				
	10	1	5					1	10		
	13	1	5−			1	11−	1	11−		
6	2	5	6			5	9+				
	4	1	6			1	10−	1	III 9+		
	7	1	6			1	10+	2	10		
	14							1	11−		
Number of cases		45		8		15		20		7	

The gland in the first host thus served as a control for the partner gland in the second host. At the desired time, both hosts were killed simultaneously, the two glands dissected and compared. Twenty-three such pairs are available;

they are recorded in Table III. As in the previous experiments we find the salivary glands unable to develop when transplanted alone, while the partner glands with added ring gland implants continue development. Figure 5 of plate III illustrates such a paired transplantation. This pair was transplanted at stage 6 and left for two days in the adult host. The partner (*a*) transplanted alone, has become greatly inflated, yet has not developed beyond the stage of transplantation. The other partner (*b*) which was transplanted together with two ring glands has continued its development and has reached stage 9+. Plate III, Figure 5b illustrates clearly the characteristic large nuclei at this stage which stand out against a lightly stained background (Cf. Plate III, Figure 5b with stage 9 of the normal developmental series). The chromocenter has become invisible in at least two-thirds of the gland. The chromosome strands are broad and the chromatin bands within them very distinct. The single cells are extremely large, due to an increase in the volume of the cytoplasm. In the living condition the cytoplasm is opaque in contrast to the transparent

TABLE III

Paired transplantation of salivary glands of different age into the abdomen of adult male flies. Note: one host carries salivary gland alone, while second host carries partner salivary gland and ring gland grafts.

Stage of transplanted salivary gland	Number of ring glands transplanted	Days graft remains in host	Condition of transplanted salivary gland	
			Partner without ring gland stage:	Partner with ring gland stage:
2	4	5	2	4+
3	2	6	3	I 7+
3	2	9	3	II 8
3	2	9	3	II 9
3	2	13	3	10
4	3	1	4	5+
4	3	4	4	I 8−
4	3	7	4+	II 8+
4	3	7	4	III 9+
5	2	6	5	II 9+
5	2	9	5	10−
5	2	9	5+	10+
5	2	13	5	11−
6	2	2	6	9+
6	2	2	6	9+
6	2	2	6+	9+
6	2	2	6	9+
6	2	2	6	9+
6	2	2	6	9+
6	2	3	6	9+
6	2	4	6	10−
6	2	6	6	10+
6	2	7	6	10+

appearance of the partner gland. Moreover, the salivary gland which has undergone development is not swollen as is the case with the partner gland. This fact can be seen in all salivary glands which are transplanted together with ring glands. The ring gland must hence interfere with the secretory function of the salivary gland. On turning again to Table III we find that the first response to the ring gland is obviously a growth response. This becomes evident if one compares glands of the same age which were left for increasingly longer periods in the host. (See Tables II and III.) For example, glands transplanted at stage 4 grow in one day to stage 5+, in four days to stage 8− and reach stage 9+ when left for seven days in the host. After growth has continued for a certain length of time, the physiological condition of the glands must have changed in some way, since they now respond with metamorphosis to the ring gland factor. The younger the glands are at the time of transplantation, the later they respond with metamorphosis, for glands transplanted at stage 2 develop in five days only to stage 4+, while glands transplanted at stage 4 show indications of metamorphosis after four days. Metamorphosis is far advanced in four days when glands of stage 6 are used for the transplantation. The transplanted glands, especially the younger ones, metamorphose precociously, for the opaque metamorphosis condition is noticeable in transplanted glands as early as stage 7+, thus at a considerable earlier stage than in normal development. (See also Table II.) This abnormal relationship between the stage of development and the onset of metamorphosis becomes less pronounced as increasingly older glands are used.

The different responsiveness of younger and older salivary glands has been demonstrated very clearly in a further experiment especially designed for testing this point. Two salivary glands, one of stage 3 and the other of stage 5, were transplanted together with two ring glands into the abdomen of a single adult host. This series consisted of four hosts, each of which contained a young and an old salivary gland, as well as two ring glands. Six days after the operation, the first host was examined. The young salivary gland had developed to stage 7+ and was slightly opaque (I), while the older gland was at stage 9+ and distinctly more opaque. In two hosts examined nine days after the operation, the younger glands were found to be at stage 8 to 9 and the older glands at stage 10+. While the younger glands appeared intensely opaque, the older glands were obviously in an advanced state of metamorphosis, as indicated by large milky regions of degeneration. The last host examined 13 days after the operation revealed the young gland at stage 10 and the older one at stage 10+. Again histolysis had progressed further in the older gland.

In a second series of this kind, consisting of six animals, salivary glands of stages 2 and 3 were transplanted together with three ring glands into a single host. The results of these experiments are essentially the same as in the foregoing series; they show again that older glands are always further metamorphosed than young ones, although, as pointed out before, the onset of metamorphosis in the young glands is premature, relative to their state of development.

It has been seen that the development of the salivary gland in the adult host with the support of the ring gland is considerably slower than in normal development. However, the question as to how much development is slowed down has not as yet been examined. In order to elucidate this point, one partner of a pair of glands of stage 5 was transplanted into the abdomen of an adult host together with 3 ring glands, while the other partner was transplanted into the abdomen of an old larva shortly before pupation. In this way it was possible to make a direct comparison of the same gland in an adult and larval environment. From six such experimental pairs, five were examined two days after the operation. At this time the larval hosts pupated. In one pair the gland in the larval host was found to be at stage 10, showing clear regions of degeneration, while the partner gland in the adult host had only developed to stage 9 and appeared but slightly opaque. In two other pairs only the proximal part of the gland in the pupated larval host could be found, since the distal gland region was completely histolysed. The partner glands in the adult host in one of these pairs had developed to stage 9, being slightly opaque, while the gland in the other adult host had reached only stage 8 and was still transparent. The glands in the pupated larval hosts of the two remaining pairs could not be found, presumably because they were already completely histolysed. The partner glands in the adult hosts were found to be transparent and had reached stage 8. The last pair of this series was examined seven days after the operation. At this time the larval host had already emerged. Its graft could not be found and was apparently completely histolysed. The partner graft in the adult host was at stage 10−, showing advanced signs of histolysis.

The results of these experiments bring into focus one interesting point. We have seen from the previous experiments that growth and metamorphosis of the salivary gland is controlled by the ring gland. The salivary glands in the larval hosts are under the influence of only one ring gland, namely that of the host, while in the adult hosts the glands are exposed to the effects of two ring glands. In spite of this, we find the progress of development of the salivary glands much more rapid in the larval than in the adult host. This shows that the ring gland factor is more effective in a larval than in an adult environment.

Before closing the experimental part of this paper it should be mentioned that the ring gland factor is not species-specific, since ring glands of melanogaster cause the development of virilis salivary glands in both virilis and melanogaster hosts. Conversely, melanogaster salivary glands develop under the influence of virilis ring glands in virilis or melanogaster hosts.

DISCUSSION

The present data prove without doubt the necessity of the ring gland for the metamorphosis of the salivary gland. The responsible ring gland factor is presumably hormonal in nature, since the transplanted ring glands have no contact with the test organs which are nevertheless compelled to metamorphose. The ring gland is thus the source of the metamorphosis hormone in Drosophila and is not responsible only for puparium formation as previously assumed. In fact, puparium formation has to be considered as the first step in metamorphosis. The observations of Hadorn and Neel (1938) that ring glands implanted into young larvae bring about premature puparium formation but have no effect on the metamorphosis of the larval organs can be explained by the incompetence of the young organs to respond to the metamorphosis hormones. This is supported by the observation that young salivary glands react more slowly to the ring gland hormone than older ones and that the salivary glands must reach a certain developmental stage before they are able to respond to the metamorphosis hormone. The inability of organs to respond with metamorphosis before a certain stage of development is reached is not peculiar to salivary glands but has been observed for eye discs (Bodenstein, 1939a and b), and for ovaries (Vogt, 1941). Inasmuch as the time at which larval glands and imaginal gland anlagen reach their state of responsiveness is concerned, we find that both structures become responsive at about the same time. This, however, is true only of grafts into larval hosts; in adult hosts we find the larval glands able to metamorphose, but not the imaginal glands, in spite of the presence of several ring glands. This indicates clearly that the larval glands respond much more readily than the imaginal glands. In comparing the ring gland action in a larval and an adult environment, we find the hormone more active in the former. Whether one ring gland in the larval host is able to produce more hormone and thus assure a higher hormone concentration than four ring glands in an adult host is questionable, but would explain easily why in the adult host the imaginal anlage is unable and the larval gland is able to respond; and why in the larval host both glands are able to metamorphose synchronously. On such an hypothesis the low hormone level in the adult host is able to induce metamorphosis only in the readily responsive larval gland but not in the imaginal gland anlage, which needs a higher hormone concentration for the same task. In the larval environment on the other hand, where the hormone concentration is presumable higher, it is sufficient to induce metamorphosis in the imaginal gland also. In the light of these considerations it becomes evident that any difference in the competence of the reactive tissue might be detectable only when low hormone concentrations are used. The quantitative action of the metamorphosis hormone has also been demonstrated by Hadorn and Neel (1938), who found that pupation in Drosophila occurs sooner when three instead of one ring gland are implanted into the larval host. The question of hormone concentrations was not directly tested in our experiments because there seemed to be no difference in the effect of two or more ring glands. There is, however, some indication that one ring gland is not as effective as four; this can be seen if one compares (Table II) salivary glands which were transplanted at stage 4 together with one and with four ring glands and which were then left for six days in the adult host. The salivary glands (two cases) transplanted together with one ring gland have developed to stage 7 and the salivary glands transplanted together with four ring glands to stage 10 (three cases). Similar experiments on other organ discs not reported here gave the same results. Thus it seems quite certain that the hormone concentration is a decisive factor in the development of the salivary gland.

One of the most important facts revealed by the present investigations is that the first salivary gland response to the ring gland hormone is a growth response. It is only after the salivary glands have grown to a certain size that the hormone elicits the metamorphosis response in the salivary gland. The younger the salivary glands are at the time of transplantation the longer the growth period persists. In other words, younger glands begin their metamorphosis later than older glands when under the influence of the same number of ring glands, yet the onset of metamorphosis of young glands in adult hosts under ring gland influence is definitely premature when compared with normal development. However young the salivary glands are when transplanted, the onset of their metamorphosis is never earlier than stage 7−, as compared with stage 9 in normal development. This shows that the young glands are able to respond with growth only they have reached stage 7−. This observation leads us directly to the question why in normal development the onset of metamorphosis takes place at stage 9 and not at stage 7−. The answer to this may be found in the following considerations. The experiments have shown that the growth of the salivary glands depends on the ring gland hormone. This must obviously also be the case in normal development. Now in normal development the salivary gland is under the influence of the ring gland during the entire larval period. However, the ring gland of younger larval stages is much smaller than the ring gland of mature larvae and thus probably produces much less hormone. The hormone concentration of younger larvae is consequently expected to be much lower than in older larvae. The hormone level in younger larvae, we might argue, is thus not high enough to assure metamorphosis, but is sufficient to promote growth. As development proceeds, the ring gland grows and thus produces more hormone; at the same time however, we find the competence of the salivary gland changing. At a given time, therefore, when hormone level and responsiveness of glands are in a definite relationship with each other, metamorphosis occurs. In normal development this time is reached when the salivary glands have developed to stage 9. We may now ask why, under experimental condition, we are able to metamorphose glands which have reached only stage 7−. The answer to this lies very probably in the fact that we have changed the hormone concentration. In the adult host the hormone level produced by several ring glands is presumably much higher than in younger larvae and is thus able to induce metamorphosis in glands as young as stage 7−. Since metamorphosis proceeds at a very slow rate, we must conclude that the experimentally established hormone level in adult animals, while higher than in younger larvae, is not as high as in full-grown larvae, where young glands metamorphose much more rapidly. Whether the ring glands produce less hormone in the adult fly, or whether some other factors are responsible for the fact that we are unable to raise the adult hormone level to the equivalent of the level existing in the old larvae is, however, still questionable.

There is one further point of considerable interest. We have seen on page 22 that old salivary glands transplanted into younger larvae metamorphose before the glands of the host larvae show any signs of metamorphosis. Since the cell size of the grafted glands was found to be not larger than normal, there seems to be a limit beyond which nuclear growth is impossible. The question now arises, what exactly do we mean by this limit? Judging from the results cited earlier, we have to assume that the salivary glands become more and more responsive to metamorphosis as they increase in age. The older gland in the young host is therefore at a much more advanced stage of responsiveness than the host glands, and is hence able to react with metamorphosis to a hormone level much lower than that needed for the same reaction by the younger gland. In assuming, as we have, that the hormone level increases gradually during the larval period, the growth limit is nothing more than the expression of a definite relationship between cell competence and hormone level. In the light of this it is theoretically possible to obtain cells larger than normal, when older glands are affected by a hormone level too low for metamorphosis but high enough for growth; granted, however, that a hormone level low enough for a highly responsive gland could be found.

Logically this conception is based on the assumption that the ring gland produces its hormone during the entire larval life. Only with the demonstration of this does the present hypothesis become meaningful. Actually there is really good evidence available which shows that ring glands from larvae as young as the first instar are able to promote growth in certain organ discs. More precise information concerning this point will be given in a later communication.

SUMMARY

1. The normal development of the salivary gland of *Drosophila virilis* is described. Eleven successive stages of development have been distinguished.

2. Larval salivary glands of different ages were transplanted into the abdomens of older larvae and thus exposed prematurely to the metamorphosis factor. It was found that the metamorphosis of the transplanted glands is not autonomous but depends upon some factor in the host.

3. Glands as young as stage 3 are unable to react to the metamorphosis factor and persist as larval tissue in the adult fly. However, glands transplanted at stage 5 metamorphose synchronously with the host and hence undergo a premature metamorphosis. In these cases the transplanted larval glands are completely histolysed, and the simultaneously transplanted anlagen of the imaginal salivary gland differentiate into adult salivary glands.

4. Salivary glands of older larval donors transplanted into younger hosts metamorphose before the host glands show any signs of metamorphosis.

5. Larval salivary glands of various ages were transplanted into the body cavity of adult male flies. The thus transplanted glands ceased to grow and remained unchanged even when left for a considerable length of time in their adult environment. If, however, ring glands of old larvae are transplanted together with salivary glands into the adult host, the growth of the salivary glands is restored, leading finally to metamorphosis. These facts have been demonstrated very clearly by using salivary glands of a single donor and transplanting one partner into one host without ring glands and the other partner into a second host together with ring glands.

6. The number of ring glands implanted is of no great importance for the development of the salivary glands, since two ring glands have about the same effect as four ring glands. However, one ring gland is presumably somewhat less effective than four ring glands.

7. The rate of metamorphosis of the salivary gland in adult hosts is decidedly slower than in normal development.

8. Younger salivary glands metamorphose later than older salivary glands under the influence of the same number of ring glands.

9. Although the young glands metamorphose later, their metamorphosis is premature as far as their state of development is concerned.

10. A comparison of the time of metamorphosis of salivary glands in adult and larval hosts shows that metamorphosis proceeds much more rapidly in larval hosts in spite of the fact that in the adult host the salivary glands may be under the influence of as many as four ring glands.

11. The ring gland factor is presumably hormonal in nature, and is not species-specific.

12. The role of hormone concentration and tissue competence in the determination of the various stages of growth and differentiation in the development of the salivary glands is discussed.

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HORMONES AND TISSUE COMPETENCE IN THE DEVELOPMENT OF DROSOPHILA

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The larvae of Drosophila molt twice, and change with the third molt into the pupal stage, during which the larval organism is gradually made over into the final adult insect. It was found (Bodenstein, 1936) that the initiation of pupation depends upon some factor in the anterior part of the larva which becomes active shortly before pupation and which is presumably hormonal in nature. Hadorn (1937) has located and analyzed this factor more precisely. He brought forward conclusive experimental evidence that a hormone causing pupation in Drosophila is produced by the ring gland, a small organ of internal secretion situated dorsally between the two brain hemispheres of the larvae. Although responsible for pupation, the ring gland was seemingly unable to initiate further pupal development, i.e. the differentiation of the larval organ anlagen to imaginal completion. For larval abdomens which, as a result of the removal of the anterior part, remain constantly larval could be caused to pupate when one or more ring glands were transplanted into them (Hadorn and Neel, 1938). Yet only puparium formation but no further development could be induced. Likewise, transplantation of several ring glands into younger larvae brought about only precocious puparium formation but again no subsequent development (Hadorn and Neel, 1938). In the light of these facts it appeared highly probable that some other hormone than that for puparium formation governed imaginal differentiation. The following observations seem to verify this assumption. The imaginal differentiation of pupal abdomens proceeds to imaginal completion when the anterior pupal part is cut off about 20 hours after pupation but the abdomen remains pupal when the anterior part is removed earlier (Bodenstein, 1938 and 1939a). The imaginal differentiation of organ anlagen, for example, eye discs, also depends upon this factor in the anterior part. On the basis of this rather indirect evidence a special differentiation hormone was postulated (Bodenstein, 1938). However, attempts to localize this factor in the anterior pupal part failed completely (Bodenstein, 1939a and c). Pupal abdomens, the anterior part of which was cut off before the imaginary hormone was released and which consequently were expected to remain pupal, continue their development to imaginal completion when placed in a pure oxygen atmosphere (Bodenstein, 1939c). This observation made the existence of a special hormone for differentiation very doubtful. Moreover it was shown (Bodenstein, 1939c), that the inability of the pupal abdomen to develop could be correlated with disturbances in the development of the tracheal system. These experiments, then, indicated that abnormalities in the functional development of the tracheal system rather than the lack of a special hormone was the cause of the inability of the abdomens to develop. While these considerations do not disprove the existence of a differentiation hormone completely, they make its assumption quite unnecessary. The main object of this paper is to bring forward more conclusive evidence that the ring gland is responsible not only for pupation but also for differentiation. Actually we have to consider pupation as the first step in the process of imaginal differentiation.

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* Fellow of the John Simon Guggenheim Memorial Foundation.

Material and Methods

The experiments reported here were performed on *Drosophila melanogaster* (Ore. R+) and *Drosophila virilis* (wild stock). Both of these species were used as donors and hosts. Various organ discs were transplanted into the body cavity of adult flies and the development of the transplant in its new environment was studied. This new method (see Bodenstein, 1943b, in press) of using the body cavity of adult flies as a culture medium for larval tissues proved to be very successful and was used throughout this investigation. The mortality rate in these experiments was negligible. All the experimental animals were kept at a constant temperature of 25° ± 0.5° C.

I am greatly indebted to Dr. L. C. Dunn and Dr. Th. Dobzhansky for many stimulating discussions and for their continued interest in this work. I also wish to thank Mrs. E. Sansome for helpful criticism during the preparation of this paper.

Experiments

When larval eye or leg discs of Drosophila are transplanted into the body cavity of adult flies, the grafted organ is unable to develop. Although left for many days in the adult environment the graft remains unchanged as far as its morphological appearance is concerned. This observation is in agreement with earlier experiments of this kind (Bodenstein, 1938, p. 497). From this it was assumed that the adult environment is a medium unsuitable for the development of larval organs. It was therefore rather unexpected when it was found that larval eye discs which were transplanted simultaneously with two ring glands of mature larvae into adult flies had grown well beyond their original size. This experiment was repeated as follows: Eye discs of melanogaster donor larvae of equal age were transplanted either alone or together with ring glands into adult melanogaster hosts. Three days after the operation the grafted eye discs were dissected and compared. It was found that the eyes in hosts with ring glands were larger by far than those which were in hosts without ring glands. A great number of similar experiments was then performed, consisting of 243 cases where organ discs were transplanted together with ring glands into adult hosts and 156 control cases where the organ discs were transplanted alone. The bulk of this material comprises many different series; the series varied as to the time the organs were allowed to remain in the adult host, the number of ring glands transplanted into one host, the kind of organ disc used (eye and leg) and the kind of host used (melanogaster and virilis). In comparing the discs in hosts with and without ring glands it was invariably found that the organ discs in the hosts with ring glands had become much larger than the control discs in the hosts without ring glands. Further proof for the initiation of growth by the ring gland was obtained by experiments in which the two partners of a single organ pair were compared one with the other. For this a pair of eye or leg discs was dissected from a single donor larva and one partner disc transplanted into one host together with two to four ring glands, and the other partner transplanted alone into a second host. The results of these experiments consisting of 34 individual pairs are summarized in Table I, where it can be seen that in each pair the disc which was transplanted together with ring glands had become larger than its partner. Figure 1 (*a*, *b*) illustrates very clearly the enormous size difference between two partner discs which were dissected and photographed five days after the operation. This particular pair is a melanogaster leg pair. One partner disc (*a*) was transplanted alone and the other

TABLE I

Paired transplantation of eye and leg discs into two adult hosts. One host receives disc alone, while other receives partner disc and two to four ring glands.

Transplant	Number of pairs	Days pairs remain in hosts	Number of pairs where the disc is larger in hosts with ring glands
mel. eye	3	2	3
mel. eye	1	3	1
mel. leg	4	3	4
mel. eye	3	4	3
viril. leg	2	4	2
mel. leg	2	5	2
viril. leg	8	5	8
mel. eye	1	6	1
mel. eye	1	8	1
mel. leg	1	8	1
viril. leg	3	9	3
viril. leg	1	12	1
viril. leg	1	14	1
viril. leg	3	16	3

PLATE I

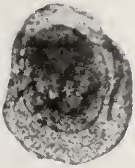
FIGURE 1. Melanogaster leg disc pair five days after the operation. One partner disc (*a*) was transplanted into an adult melanogaster host; the other partner disc (*b*) into an adult melanogaster host together with four ring glands. Note: the enormous difference in size between the two discs.

FIGURE 2. *b*: virilis leg disc four days after the operation, showing the first signs of metamorphosis, i.e. the beginning of evagination. The disc was transplanted together with four ring glands into an adult virilis male host. *a*: Virilis leg disc at the time of transplantation. *c*: a normal pre-pupal leg disc beginning to evaginate. Note: the similarity in the process of evagination between the transplanted (*b*) and the normal (*c*) leg.

FIGURE 3. A virilis leg disc transplanted together with three ring glands into an adult virilis female host, 13 days after the operation. The leg disc is completely differentiated. Note: the well-formed dark brown chitinous tarsus segments, with hairs and claws well developed.

FIGURE 4. Virilis leg disc pair transplanted into two adult virilis male hosts six days after the operation. Note: same size of both discs.

FIGURE 5. Virilis leg disc pair four days after the operation. One partner disc (*a*) was transplanted into an adult virilis male host and the other partner disc (*b*) into an adult virilis female host. Note: disc in female host (*b*) has become much larger than the partner disc (*a*) in the male host, which has not grown at all.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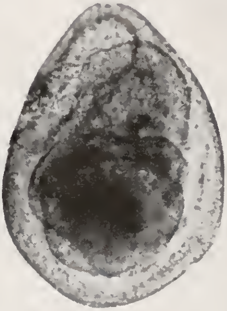
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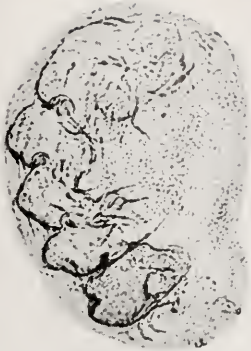
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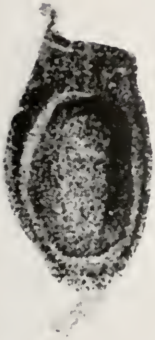
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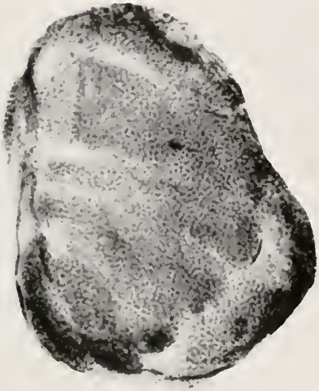
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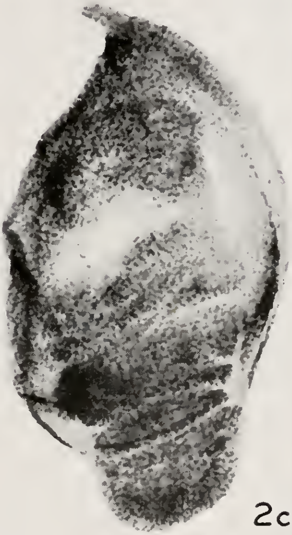
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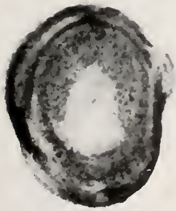
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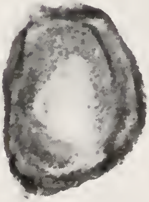
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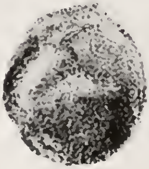
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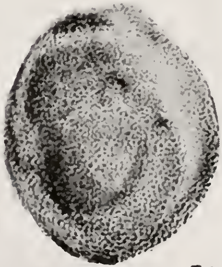
5b

PLATE I

partner (*b*) together with four ring glands into the abdomen of adult melanogaster hosts.

In normal development we notice that a short time after puparium formation many organ discs undergo a characteristic change of form; they evaginate and obtain thus their typical pupal shape. This evagination process is one of the first visible signs of metamorphosis of the organ discs. Now we find that the transplanted leg discs in the body cavity of adult flies also evaginate under the influence of the ring gland after they have grown to a certain size. This induced evagination process is not quite complete, presumably because of mechanical difficulties, but is nevertheless very clear. This is illustrated in Figure 2 (*a*, *b*), Figure 2*a* shows a leg disc at the time of transplantation; Figure 2*b* a leg disc of a normal young pupa which has started to evaginate, and Figure 2*c* a disc which was transplanted into an adult host together with ring glands and which was dissected four days after the operation. In comparing Figure 2*b* with Figure 2*c* one may notice the similarity between normal and induced evagination. This observation clearly proves that the ring gland is able to induce the first stages of metamorphosis in the transplanted leg anlage. Moreover the ring gland is able to induce complete metamorphosis in the leg, if the leg is left in the adult host long enough. In these cases we find a completely differentiated imaginal leg with femur, tibia and tarsus segments as well as well-formed and dark brown chitinized hairs, bristles and claws in the abdominal cavity of the fly (Figure 3). From these experiments it becomes evident that the ring gland is not only responsible for an early initiation of growth, but also for the imaginal differentiation of the organ discs.

While it is true that organ discs transplanted together with ring glands were always larger than the control discs transplanted without ring glands, there was nevertheless a certain variability in the growth of the discs. In some cases where the discs were transplanted alone into adult hosts it was found that they had not grown at all, although they had remained for ten days or longer in these hosts. In other cases the discs had grown quite well and even showed signs of metamorphosis although no ring glands were present. Similarly, discs of equal age grown for the same length of time in the presence of the same number of ring glands could vary quite extensively in size. Now it has to be realized that in experiments of the kind described one deals with three different developmental systems which together determine the outcome of the experiment. These systems are: 1. The adult host environment; 2. The activating system, i.e. the ring glands; 3. The reacting system, i.e. the test organ discs. Thus in order to clarify the observed discrepancies in the experiments, a more thorough investigation of these three systems was undertaken. For this purpose experiments were designed in such a way that two of the systems were held constant and the third one varied. In doing this for each system in turn a clear understanding of the part played by each system was obtained.

1. The adult host environment

Under this general heading we will discuss a number of experiments in which the adult flies used as hosts were varied. As an indicator for possible differences between the various hosts we used only the early growth reaction of the test organs. This method is very sensitive, for even small differences reflect themselves very clearly in the growth of the test organ discs, especially when the two partners of a single organ pair are compared.

a. Growth variability test in male hosts without ring glands.

This series was designed to test whether there is any difference in the growth of test organs in different host individuals of the same sex. For this purpose, pairs of leg discs were dissected from virilis larvae and one partner disc transplanted into one and the other partner disc into a second adult virilis male host. The two hosts were then reared and dissected together. From eight such pairs two pairs were dissected two days, one pair three days and five pairs five days after the operation. The partner discs in all pairs were found to be the same size (Figures 4*a* and 4*b*). This proves that there is no detectable difference in the environment of the different individual male hosts as far as the test organs are concerned. Moreover, the leg discs remained unchanged in size, which indicates that no growth had occurred from the time of transplantation until they were dissected five days later. In order to obtain more information on this point, four of these disc pairs were again transplanted into adult male hosts. Seven days later they were dissected and found to be unchanged. Thus we must conclude that the virilis organ discs are unable to grow in an adult virilis male environment.

b. Growth tests in hosts of different age.

The question whether there is any difference in hosts of different age has been tested in the following way. The two partners of a pair of virilis leg discs were transplanted, one into a one-day old virilis male host and the other into a 29-day old virilis male host. From five such pairs one was dissected four days, and four pairs six days after the operation. In all cases it was found that the partner discs of the single pairs were of the same size. There was also no growth in either partner discs during the time they remained in the hosts.

In a second series consisting of six pairs, the discs were transplanted into female hosts instead of into male hosts. One female host was two days old and the other 30 days old. One pair, dissected three days and five pairs dissected five days after the operation revealed again that the two discs of one pair were of equal size. Yet in contrast to the previous series each of the discs had grown during the time it remained in the host.

These experiments prove that there is no difference between young and old hosts. They confirm the previous observation that the environment of each individual male host is the same, and extend the information in showing that this is also true for the environment of each individual female host. The observation that no growth takes place in discs transplanted into male hosts is also confirmed. However, when one compares female and male environment one finds the discs able to grow in the former but not in the latter environment.

c. Growth tests in male and female hosts.

It is evident from the foregoing experiments that male and female environments are different as to their effect on the growth of the grafted organ discs. Decisive evidence for this is provided by the following experiments. The two partners of the virilis leg disc pairs were transplanted, one into a male, the other into a female virilis host. Five such pairs were dissected three days, and eight pairs four days after the operation. In all pairs it was found that the discs in the female hosts were much larger than their partner discs in the male hosts, which had not grown at all. Figure 5 (*a*, *b*) illustrates this effect very clearly.

Similar results were obtained in another series of experiments (five pairs dissected three days after the operation), where leg discs pairs of virilis were transplanted into female and male melanogaster hosts.

In transplanting the two partners of virilis eye disc pairs into virilis male and female hosts (three pairs dissected three days after the operation), we again find the discs in the female hosts larger than their partners in the male hosts (Figures 6*a* and 6*b*). The eye discs in the male hosts had ceased to grow, being of the same size at the time of dissection as at the time of transplantation.

Finally, in a last series of this kind, the two partners of a pair of salivary glands of virilis larvae were transplanted, one into a male and the other into a female virilis host. From eight such pairs three were dissected in four days, two, seven days and three, eight days after the operation. Again, as in the case of the organ discs, it was found that the glands in the male hosts had not developed while their partners in the female hosts were all in an advanced stage of development.

d. Growth test in different host species.

The object of this group of experiments was to test for possible species differences between virilis and melanogaster hosts. To this end the two partner discs of a virilis leg pair were transplanted, one into a virilis male host and the other into a melanogaster male host. From six pairs available, two were dissected three days, two, four days and two, six days after the operation. The transplanted discs were found to be of the same size in both hosts in all pairs. There is evidently no difference between the melanogaster and virilis environment, as far as it affects the graft.

In a second series of experiments, comprised of seven pairs, which were dissected three days after the operation, the two virilis leg pair partners were transplanted into a melanogaster and a virilis female host. Being in a female environment, the discs in both hosts had, of course, grown; in one pair the discs were of the same size, in three pairs the discs in the melanogaster hosts were

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PLATE II

FIGURE 6. Virilis eye disc pair three days after the operation. One partner disc (*a*) was transplanted into an adult virilis male host and the other partner disc (*b*) into an adult virilis female host. Note: disc in female host has become much larger than partner disc in male host, which has remained unchanged.

FIGURE 7. Virilis leg disc pair three days after the operation. One partner disc (*a*) was transplanted together with two ring glands into an adult virilis male host and the other partner disc (*b*) was transplanted together with two ring glands into an adult melanogaster male host. Note: disc (*b*) in the melanogaster host has become much larger than its partner (*a*) in the virilis host.

FIGURE 8. Virilis leg disc pair four days after the operation. One partner disc (*a*) was transplanted into an adult virilis male host together with four ring glands and its partner (*b*) into an adult virilis male host together with eight ring glands. Note: both discs have grown the same.

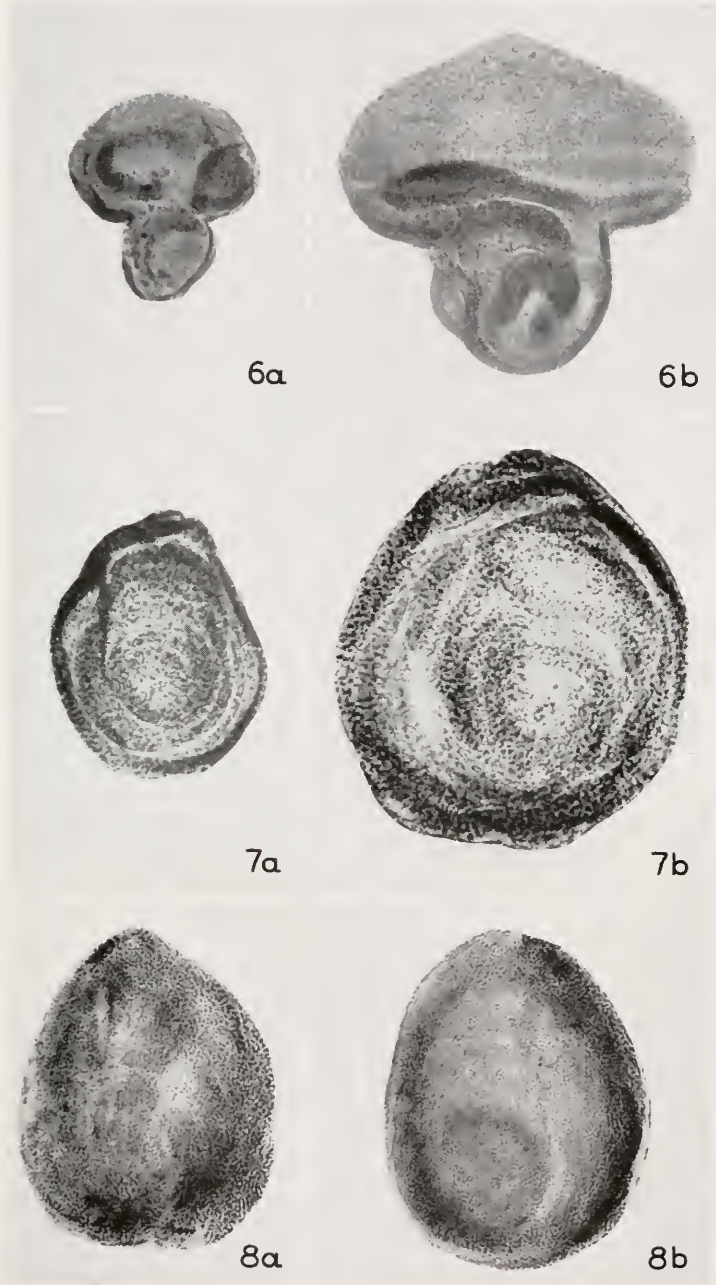
6a
6b
7a
7b
8a
8b

PLATE II

slightly larger and in the last three pairs they were somewhat larger in the virilis hosts. The fact that the discs may be larger in melanogaster female as well as in virilis female hosts indicates that there is no significant difference between the environment of both host species.

e. Growth test in male and female hosts in the presence of ring glands.

It has been shown before that organ discs grow larger in female hosts than in male hosts. The question now arises, how the growth of equal discs is affected when they are under the influence of the same number of ring glands in both environments. For this, two partner discs of a virilis leg pair were transplanted, one into a virilis male and the other into a virilis female host, while at the same time each of the two hosts in two of such pairs received five ring glands, and in two other pairs four ring glands from mature larvae. In dissecting the two pairs with five ring glands three days, and the two pairs with four ring glands four days after the operation, it was found that the discs in the female hosts of all pairs were larger than their partner discs in the male hosts. However, in contrast to the earlier experiments, where the discs were grafted alone, into male and female hosts, both discs in this experimental combination had grown. Moreover, the discs in the female hosts had become much larger in the presence of ring glands than discs which had grown in female hosts without ring glands.

f. Growth tests in different host species in the presence of ring glands.

If the experiments where virilis partner leg discs are transplanted into two hosts of different species (melanogaster male and virilis male) are repeated, but each host receives in addition two ring glands from mature larvae, then the results obtained are quite different. The transplanted organ discs are much larger in the melanogaster hosts (Figures 7*a* and 7*b*). This was observed in nine out of 12 pairs four of which were dissected three days, six four days and two six days after the operation. Only in three pairs left for four days in the host were the discs found to be alike in both hosts. Moreover in another pair, where one partner disc was transplanted into a virilis male host together with five ring glands, and the other partner disc into a melanogaster male host together with four ring glands, it was found again that the disc in the melanogaster host was much larger. Finally, in two additional pairs in which one partner disc was transplanted together with four ring glands, into a virilis male host, and the other partner into a melanogaster male host together with only two ring glands, it was again observed that, three days after the operation, the discs in the melanogaster hosts were much larger than their partners in the virilis hosts. Thus two ring glands in melanogaster hosts are able to induce more growth in the test organs than four ring glands in virilis hosts. Since in all these cases virilis ring glands were used as grafts we witness the peculiar fact that virilis ring glands are more effective in a foreign than in their own species environment.

II. The activating system

Experiments described in this section are designed to further the understanding of the ring gland action.

a. The effect of different numbers of ring glands on organ growth.

We have seen that organ discs transplanted into adult male hosts are able to grow only when under the influence of simultaneously transplanted ring glands. It remained to be shown, however, as to how many ring glands are actually needed to assure maximum growth of the organ disc. For this, eye discs of virilis larvae of equal size were transplanted from virilis male hosts while in addition, each of these hosts received a different number of ring glands from mature larvae. The results of the experiments are summarized in Table II, where it can be seen that two ring glands produce a greater growth effect in the test organs than one ring gland, but that the effect of four or eight ring glands is the same as that produced by only two ring glands. This evidence is further supported by experiments in which the two partners of single virilis leg pairs subjected to the influence of a different number of ring glands in virilis male hosts are compared. Table III summarizes the results obtained from six such pairs, showing again that the growth of the test organ is the same whether two, four or eight ring glands are present (Figures 8*a* and 8*b*). In summarizing the results, we must conclude that one ring gland is apparently unable to raise the hormone concentration in the adult male fly to a level high enough to assure maximal growth in the test organs. Yet the hormone concentration produced by two ring glands must have reached the level of saturation as far as the growth

TABLE II

Effect of different number of ring glands on eye growth.

Experiment	Number of ring glands	Transplant remains in hosts for three days		Transplant remains in hosts for four days	
		Number of cases	Size of eyes	Number of cases	Size of eyes
A	0	4	no growth	4	no growth
B	1	4	larger than A	4	larger than A
C	2	4	larger than B	4	larger than B
D	4	4	larger than C	4	larger than C
E	8	4	larger than C	4	larger than C

TABLE III

The effect of different numbers of ring glands on leg growth.

Number of ring glands compared	0 and 1	2 and 4	4 and 8	2 and 8	2 and 8	2 and 8
Days leg pairs remain in hosts	4	4	4	3	9	9
Size of leg pairs	larger in ring gland host	same in both hosts	same in both hosts	same in both hosts	same in both hosts	same in both hosts

of the test organ is concerned, since more than two ring glands have no greater effect than only two ring glands.

Evidence that the number of ring glands is also of importance for the time of imaginal differentiation of the test organ is provided by the following experiments. Melanogaster eye discs of equal size were transplanted into adult melanogaster female hosts, (*a*) alone, (*b*) together with two ring glands, (*c*) together with three ring glands, (*d*) together with four ring glands. All hosts were dissected eight days after the operation. It was found that in the hosts without ring glands the eye discs had grown, but were still white and showed no sign of imaginal differentiation (seven cases). In the hosts with two ring glands, reddish pigmented regions could be seen in the transplanted discs, showing that the ring gland had brought about pigment differentiation in the eye disc (six cases). The eyes in hosts with three ring glands (four cases) and in hosts with four ring glands (four cases), had developed to the same stage of pigmentation. In a further set of experiments melanogaster eye discs were transplanted alone into melanogaster female hosts but left for 16 days (two cases), 17 days (one case), and 22 days (one case), in the hosts before they were dissected. By this time pigmentation had also started in these eyes but was in a much less advanced stage of development. This is indicated by the slight yellow coloration, as contrasted with the reddish color, developed in eyes grown in hosts with ring glands. These experiments show that color development can take place in eye discs transplanted into female hosts without the support of ring glands, but that it is much less rapid than in female hosts in the presence of ring glands. The onset and degree of eye pigmentation in the presence of two, three or four ring glands in female hosts is about the same. Thus as in the experiments where the effect of different numbers of ring glands on the early growth of the eye discs was tested we find that for the later processes of differentiation also two ring glands produce the maximal effect.

b. The effect of ring glands of different age on organ growth.

Until now we have studied only the effects of mature ring glands, that is, of ring glands from larvae shortly before pupation. It remains to be seen, however, whether there is any difference in the effects produced by younger or older ring glands. Single pairs of virilis leg discs were thus transplanted, one partner alone and the other partner together with ring glands, into two virilis male hosts. The virilis ring glands used for each pair were of different age. In this way progressively younger ring glands were tested as to their effect on the growth of the organ discs. In two series of this kind, each host received three ring glands, in a third series, four ring glands. All pairs in the three separate series were dissected five days after the operation and the growth of the disc in each pair compared. The results of the experiments are summarized in Table IV, where it can be seen that ring glands of all ages, even when coming from larvae as young as five and a half days before pupation, i.e. young first instar larvae, are able to promote growth in the transplanted leg test disc. This proves that the ring gland can produce its growth hormone during the entire larval period of the animal. Whether there may be any interruption in the hormone production of the ring gland during this period, as the few negative cases might indicate (see Table IV), is not known, and needs further investigation.

c. Differences in hormone production of young and old ring glands.

The question as to the amount of hormone produced by young and older ring glands was tested in the following way: melanogaster leg discs of an average diameter of 13 units were divided into three lots. One set of legs was transplanted into melanogaster females alone, the legs of the second set into melanogaster females each together with one melanogaster ring gland from an old larva shortly before pupation, and the legs of the third set into melanogaster females each together with one two-day younger melanogaster ring gland. Three days after the operation the hosts of these three groups were dissected and the transplanted

TABLE IV

The effect of ring glands of various age on organ growth. In all cases the test organ remained for five days in the host. Positive indicates that the leg partner in the host with ring glands is larger than its partner in the host without ring glands; while negative indicates that both leg partners in the two hosts compared are of the same size.

Number of ring glands transplanted	Ring gland donor. Age in days before pupation	Ring gland donor, larval stage	Result
3	before pupation	3	positive
3	before pupation	3	positive
3	1	3	positive
3	1	3	positive
3	2	3	positive
3	2	3	positive
3	3	⅔	negative
3	3	⅔	negative
3	4	2	negative
3	4	2	positive
3	5	1	positive
3	before pupation	3	positive
3	before pupation	3	positive
3	1	3	negative
3	1	3	positive
3	3	3	positive
3	4	2	positive
3	4	2	negative
3	5	1	positive
3	5	1	positive
4	1	3	positive
4	1	3	positive
4	2	3	negative
4	2	3	positive
4	3	3	positive
4	4	2	positive
4	4	2	negative
4	5	1	positive
4	5	1	positive
4	5½	1	positive
4	5½	1	positive

leg discs measured and compared. The legs in the hosts without ring glands (eight cases) had grown to an average diameter of 16 units. The legs in the second group with one young ring gland (five cases) were found to average 21 units in diameter and in the last group with one old ring gland, the average diameter of the legs (four cases) was 24 units. It was noticed, moreover, that the legs in the last group had begun to evaginate, which was not the case in the other two groups.

Now one may test, although in a somewhat different way, the amount of hormone produced. If it is true, as the above mentioned experiments indicate, that one young ring gland produces less hormone than one old ring gland, one might expect equal discs transplanted at the same time, and left long enough in the adult host, to be advanced further in their metamorphosis in the presence of old ring glands than in the presence of the same number of younger ring glands. In such an experiment we use the state of metamorphosis rather than differences in growth as an indicator for the hormone concentration. In order to elucidate this point, melanogaster eye discs of equal age were transplanted into adult hosts, some with four ring glands from larvae shortly before pupation and others with four one day younger ring glands. Eight days after the operation the hosts were dissected and the discs compared. The eyes in hosts with four ring glands had developed yellow-red pigment (four cases) while the eyes in hosts with four younger ring glands were much less advanced in their differentiation. Although they had grown extensively in the presence of the younger ring glands, they were still white, showing no trace of pigmentation (four cases). From these two groups of experiments we may thus conclude with reasonable certainty that young ring glands produce less hormone than old ring glands.

d. *Species differences in ring glands.*

Qualitatively the ring glands of virilis and melanogaster are the same. This has been shown many times in experiments where the action of virilis or melanogaster ring glands has been tested as to its effect on the growth and differentiation of melanogaster or virilis organ discs. The question whether there is any quantitative difference in the amount of hormone output during a given time between the ring glands of these two species is, however, not so clear. Since the ring gland of virilis is larger than that of melanogaster one might expect it to produce more hormone. If quantitative differences between melanogaster and virilis ring glands are present, they are at least not large, as the following experiment indicates. Equal melanogaster eye discs were transplanted into adult melanogaster female hosts together with two ring glands from a melanogaster larva shortly before pupation (three cases) and together with two virilis ring glands from larvae shortly before pupation (three cases). The dissection of these cases eight days after the operation showed that in the hosts with melanogaster ring glands, one eye disc had developed slight yellow pigment and two eye discs yellow-red pigment. In the hosts with two virilis ring glands, two eye discs had become slightly yellow and one eye disc yellow-red pigmented. We thus observe about the same amount of development under the influence of the same number of melanogaster or virilis ring glands, indicating that there is no difference in the quantity of hormone production between the ring glands of these two species tested.

e. The time of action of ring glands in adult hosts.

The question as to how long transplanted ring glands in adult hosts continue to produce hormone was tested as follows: virilis ring glands from larvae shortly before pupation were transplanted into adult virilis males. The glands were left in these hosts for a certain length of time, then dissected out and re-transplanted into a second adult host together with one partner disc of a leg pair. The other leg partner was transplanted alone into another virilis male host. After several days the pair of hosts was dissected, the growth of the leg discs compared and the ring gland grafts recovered. The recovered ring glands were now grafted for the third time into a virilis male host together with new test leg discs, the partners of which were again transplanted as the growth control into virilis male hosts alone. Several days later the pairs were dissected, the leg discs compared and the retransplantation procedure of the recovered ring glands using new test organs and new hosts repeated once or twice more. Since it was not easy to recover such a small organ as the ring gland from the body of the adult fly, two or four ring glands were usually transplanted together into one host. If one ring gland was lost in the dissection, the remaining ring glands could be used to continue the test. Table V shows the results of these experiments. Each of the five columns (A to E) represents one case of successive re-transplantation of the same original glands. Now, as it can be clearly seen from Table V, it was found that ring glands after being in adult hosts for 22 days, during which time they had been three times retransplanted and found to be active, were still active in a fourth transplantation (Table V B). Similarly, ring glands which were left for 22 days in one host before they were tested in a second host for their activity were still able to induce growth in the test

TABLE V

The time of action of ring glands in adult hosts. (For explanation see text.) Positive indicates that the retransplanted ring gland has stimulated the growth of the test organ.

Experiment	A	B	C	D	E
Days ring gland remains in first host	6	6	10	10	22
Days ring gland retransplanted together with test organ remains in second host. Condition of test organ	5 positive	6 positive	6 positive	6 positive	6 positive
Days ring gland retransplanted together with test organ remains in third host. Condition of test organ	4 positive	5 positive		6 positive	
Days ring gland retransplanted together with test organ remains in fourth host. Condition of test organ		5 positive		4 negative	
Days ring gland retransplanted remains in fifth host. Condition of test organ		3 positive			

organ (Table V E). From these experiments we must conclude that ring glands transplanted into adult hosts secrete their hormone continuously for a long time.

III. The reacting system

It has to be realized that the various kinds of organ discs may differ as to their responsiveness towards the same hormone level. We may also expect differences in the responsiveness between old and young organ discs. Experiments which investigate these possibilities are presented in the following.

a. The differentiation capacity of different discs in adult hosts.

In comparing the first growth effect of such organs as eye and leg discs, one finds both very responsive to the hormone of the ring gland. Even in female hosts without ring glands, which must be considered the least favorable environment, the growth effect of both discs is considerable. Thus there seems to be no appreciable difference in the responsiveness between leg and eye discs. Yet when one compares the further development of these discs in the adult environment a marked difference between these organs becomes evident. One finds the leg discs able to differentiate in the adult host to imaginal completion under the influence of ring glands, but not the eye discs, which never continue their differentiation beyond the first stages of pigmentation. The leg discs in their final state of differentiation show typical imaginal characteristics; i.e. dark brown chitinized leg segments covered with chitinized hairs and bristles and with a blackish chitinized end claw on the distal tarsus segment. In the eye discs on the other hand, we find that the pigment is the only component which differentiates to an appreciable extent. There is however some doubt whether even pigmentation reaches its final imaginal stage. The development of hairs, bristles, lenses, or the darkening of the chitinous eye parts has never been observed in eye grafts. Although a more detailed histological examination of these partially developed eyes is still missing, there can be no doubt that differentiation is incomplete, since it would have been easy to detect chitinous structures in total mounts if they were present. It was found, moreover, that the anlage of the genital apparatus, when transplanted into adult hosts, is unable to differentiate at all, even in melanogaster female hosts in the presence of four ring glands, thus in an environment where the ring glands are most effective. Independent of the time these genital discs remain in the host, they never develop beyond a stage corresponding to the stage the discs would have reached in normal development at the time of puparium formation. In Table VI we have summarized a number of experiments in which different organ discs were transplanted together with ring glands into different adult hosts. Only such cases are recorded where the grafts were left for more than seven days in the host. We find, for example, that a melanogaster leg disc in a melanogaster female host in the presence of only one ring gland has already differentiated imaginal characters eight days after the operation, while a melanogaster eye disc in the same host in the presence of as many as four ring glands has developed only to the stage of pigment formation 19 days after the operation. Since about the same amount of pigment is present in melanogaster eyes which were left for eight days in female hosts together with two ring glands, it follows that the differentiation in the 19-day old eye has not progressed much beyond that observed in the eight-day old eye.

Thus in a melanogaster female host environment under the influence of two or more ring glands, the eye discs reach their limit of differentiation about ten days after the operation. As far as the genital discs are concerned, we find them to grow somewhat beyond their stage of transplantation. Their growth, however, is very much slower than that of leg or eye grafts, even in their early growth effect. They never surpass, as said before, a prepupal stage, although they may be as long as 17 days in a female environment under the influence of four ring glands.

TABLE VI

The differentiation capacity of different discs in adult hosts.

Number of cases	Transplanted organ	Donor	Host	Number of transplanted ring glands	Days transplant remains in host	Result
1	♀ genital disc	viril.	viril. ♀	0	8	no clear change
1	♀ genital disc	mel.	mel. ♀	0	9	no clear change
2	♀ genital disc	viril.	viril. ♀	0	17	prepupal
2	♀ genital disc	mel.	mel. ♀	0	20	prepupal
2	eye	mel.	mel. ♀	0	16	light yellow spots; no hairs
1	eye	mel.	mel. ♀	0	17	light yellow spots; no hairs
1	eye	mel.	mel. ♀	0	22	light yellow spots; no hairs
3	eye	mel.	mel. ♀	1	9	red yellow spots; no hairs
1	eye	mel.	mel. ♀	1	16	reddish spots; no hairs
1	leg	mel.	mel. ♀	1	9	brownish hairs and chitin diff.
1	♀ genital disc	viril.	mel. ♀	4	7	little growth; prepupal
1	♀ genital disc	viril.	viril. ♀	4	8	little growth; prepupal
1	♀ genital disc	viril.	mel. ♀	4	9	little growth; prepupal
1	♀ genital disc	viril.	mel. ♂	4	9	little growth; prepupal
2	♀ genital disc	mel.	mel. ♀	4	9	little growth; prepupal
2	♀ genital disc	viril.	viril. ♂	2	10	little growth; prepupal
2	♀ genital disc	viril.	viril. ♂	4	10	little growth; prepupal
1	♀ genital disc	viril.	viril. ♀	4	10	little growth; prepupal
1	♀ genital disc	viril.	viril. ♂	4	13	little growth; prepupal
1	♀ genital disc	viril.	mel. ♂	4	13	little growth; prepupal
1	♀ genital disc	mel.	mel. ♀	4	14	little growth; prepupal
1	♀ genital disc	viril.	viril. ♀	4	17	little growth; prepupal
6	eye	mel.	mel. ♀	2	8	yellow red spots; no hairs
4	eye	mel.	mel. ♀	3	8	yellow red spots; no hairs
4	eye	mel.	mel. ♀	4	8	yellow red spots; no hairs
1	eye	mel.	mel. ♀	4	16	large reddish spots; no hairs
1	eye	mel.	mel. ♀	4	19	large reddish spots; no hairs
1	eye	mel.	mel. ♀	3	23	large reddish spots; no hairs
1	leg	viril.	viril. ♂	3	9	large; no hairs
4	leg	mel.	mel. ♀	3	9	brownish hairs and chitin diff.
1	leg	viril.	mel. ♀	4	9	brownish hairs and chitin diff.
2	leg	viril.	viril. ♀	3	10	hairs diff.; still white
1	leg	viril.	viril. ♀	3	10	brownish hairs and chitin diff.
1	leg	viril.	viril. ♂	3	12	hairs diff.; still white
2	leg	viril.	viril. ♀	3	13	brownish hairs and chitin diff.
1	leg	viril.	viril. ♂	3	14	brownish hairs and chitin diff.
3	leg	viril.	viril. ♂	3	16	brownish hairs and chitin diff.

TABLE VII

Comparison of the differentiation capacity of different discs from the same donor larvae transplanted together with ring glands into one adult host.

Experiment	Transplant	Donor	Host	Number of transplanted ring glands	Transplant remains in host	Condition of organ at dissection		
						Leg disc	Eye disc	Genital disc
A	leg eye disc	mel.	mel.	3	8	large, first white hairs	red yellow spots; no hairs	—
B	leg eye disc	mel.	mel.	3	8	large, first white hairs	red yellow spots; no hairs	—
C	leg eye disc	mel.	mel.	3	8	large, first white hairs	red yellow spots; no hairs	—
D	leg eye disc	viril.	mel. ♀	4	14	brownish hairs and chitin	large white; no hairs	—
E	leg eye genital disc	viril.	viril. ♀	3	8	large, no hairs	large white; no hairs	little growth; prepupal
F	leg eye genital disc	viril.	viril. ♀	3	11	large, no hairs	large white; no hairs	little growth; prepupal
G	leg eye genital disc	viril.	viril. ♀	3	11	large with white hairs	large white; no hairs	little growth; prepupal
H	leg eye genital disc	viril.	viril. ♀	3	11	large with white hairs	large white; no hairs	little growth; prepupal
I	leg eye genital disc	viril.	viril. ♀	3	11	brownish hairs and chitin	large white; no hairs	little growth; prepupal
J	leg eye genital disc	viril.	mel. ♀	4	9	large with white hairs	large white; no hairs	little growth; prepupal
K	leg eye genital disc	viril.	mel. ♀	4	13	large with white hairs	large white; no hairs	little growth; prepupal
L	leg eye genital disc	viril.	mel. ♀	4	13	large with white hairs	large white; no hairs	little growth; prepupal
M	leg eye genital disc	viril.	mel. ♀	4	13	large with white hairs	large, yellow spots, no hairs	little growth; prepupal
N	leg eye genital disc	viril.	mel. ♀	4	11	large with white hairs	large white; no hairs	little growth; prepupal
O	leg eye genital disc	viril.	mel. ♀	4	14	brownish hairs and chitin	large white; no hairs	little growth; prepupal

The capacity of the three imaginal discs tested to differentiate in adult hosts in the presence of ring gland thus decreases in the order leg, eye and genital disc. This can be demonstrated conclusively by a somewhat different experiment, as follows: a leg, eye and genital disc from the same donor larva were transplanted into a single host, together with ring glands. In this way all the three discs are under the influence of the same number of ring glands in the same host environment and can thus be compared more directly than in the previous experiments. Fifteen such cases are shown in Table VII, where it can be seen that the differentiation capacity of eye, leg and genital discs of the same animal differs markedly under the same hormonal conditions.

Before closing this section, one further point of importance should be mentioned. It has been found that there are also differences in the differentiation capacity in the various regions of the same organ disc. The clearest example of this phenomenon is provided in the differentiation of the leg disc. While it is true that leg discs are able to differentiate to imaginal completion in adult hosts, this statement must be modified somewhat, because it applies only to the distal leg disc portions. It is known that the larval leg disc not only includes the presumptive tissue of the actual adult leg, but also some material which gives rise to the ventral body wall in the nearest neighbourhood of the leg. This proximal portion of the leg disc never differentiates completely, while the distal leg portions consisting of femur, tibia and tarsus develop to imaginal completion. The differentiation capacity of the leg parts seems to increase in a proximal distal direction, since we find the tarsus segments always to be the first structures which become imaginal, and only if the discs are left longer in the host do we find tibia and femur completely differentiated. However, we have not been able to compel the proximal leg disc portions to become completely differentiated, although the discs have remained for a considerably longer time in the host than that needed for the complete differentiation of the distal leg parts. A very similar situation prevails in the eye discs, where we find that material which gives rise to pigment cells is able to express its differentiation tendencies, resulting in the formation of well-differentiated red pigment, while the material destined to form hairs or lenses is unable to differentiate to any great extent. Moreover, there seem to be regional differences in the eye for pigment formation also, for we observe most frequently that only certain eye regions are pigmented while others are still white. It seems possible that this last phenomenon might be correlated with the position of the graft in the host as well as with the arrangement of folding of the developing eye, which in turn may affect the oxygen supply in the different eye regions, and thus promote or inhibit, as the case may be, the oxidation of pigment.

b. Differences between young and old discs.

Young and old discs in the same hormonal environment differ as to their time of differentiation. This has been shown by the following experiment. Young and old leg or eye discs were transplanted simultaneously into single adult hosts together with one or more ring glands. The grafts were left in the host for not less than nine days; they were then dissected and compared. In all cases it was found that the older graft was further differentiated than the younger graft (see Table VIII).

TABLE VIII

Differences between young and old discs, transplanted together into one host.

Experiment	Transplant	Donor	Host	Number transplanted ring glands	Days transplant remains in host	Condition of organ at dissection	
						Old disc	Young disc
A	young and old eye	mel.	mel. ♀	1	9	large reddish spots; no hairs	large white; no hairs
B	young and old eye	mel.	mel. ♀	1	9	large reddish spots; no hairs	large white; no hairs
C	young and old leg	mel.	mel. ♀	1	9	large, with white hairs	large; no hairs
D	young and old leg	mel.	mel. ♀	1	9	large, with white hairs	large; no hairs
E	young and old leg	viril.	viril. ♀	3	10	large, with white hairs	large; no hairs
F	young and old leg	viril.	viril. ♀	3	10	large, with white hairs	large; no hairs
G	young and old leg	viril.	viril. ♀	3	10	brownish hairs and chitin	large; no hairs
H	young and old leg	viril.	viril. ♀	3	13	brownish hairs and chitin	large; no hairs
I	young and old leg	viril.	viril. ♀	3	13	brownish hairs and chitin	yellowish hairs and chitin

c. The responsiveness of organs of different species.

If one compares (Tables VI and VII) the final developmental condition of melanogaster eyes with virilis eyes which were left for the same length of time in an approximately equal adult environment, one observes that the melanogaster eyes have developed further than the virilis eye discs. This indicates a difference in the competence of virilis and melanogaster eyes to respond to the same hormonal conditions. The same indication is seen in another set of experiments where virilis leg discs were transplanted into virilis female hosts together with three ring glands (five cases) and melanogaster leg discs (four cases) into virilis females together with three ring glands. Eleven days after the operation two melanogaster legs had developed hairs which were, however, still white; the two other melanogaster legs were completely differentiated, showing yellow-brown chitinous structures and hairs. Three of the virilis legs were without any hairs, one had hairs but was white, and one was completely differentiated. Although these observations speak for the assumption that virilis organs respond with greater difficulty to the same hormonal environment, there is one further point to be taken into consideration. Virilis and melanogaster differ in their time of development. The larval as well as the pupal period of virilis is much longer than that of melanogaster. Therefore it is possible that in the above-mentioned experiments, leg discs of unequal age were compared, especially since no accurate record was made of the exact age of the donor discs. The observed difference in the time of differentiation between virilis and melanogaster organs may thus not really reflect species differences but rather age differences. Even if we assume that the organs in question were of the same age, this would mean only that they were alike in their chronological age but not in their physiological age. In the light of these considerations it becomes evident that it is rather difficult in experiments of this kind to be quite sure whether any discrepancies in the time of differentiation between discs of two species are caused by species-specific responses or age effects.

DISCUSSION

The present investigations have brought forward a number of pertinent facts concerning the relationship between hormone actions and tissue competence in the development of Drosophila organ discs. It has been shown that the organ discs depend for their growth as well as for their imaginal differentiation upon the action of the ring gland which functions as a gland of internal secretion. As judged from their effect on test organs, young ring glands produce qualitatively the same hormone as ring glands of a mature larva. The quantity of hormone produced by young ring glands is presumably less than that produced by old ring glands. Equal ring glands differ as to their effect in hosts of different species and in the two sexes of the same species. The amount of organ growth during a given time and the speed at which differentiation proceeds depends upon the number of ring glands, i.e. on the amount of hormone available as well as on the competence of the organs to respond. Different organ discs as well as discs of different ages and different regions within the same organ disc differ as to their competence to respond. These facts reflect very clearly the highly relative nature of conditions which find their expression in the processes of growth and differentiation. They show that we cannot ascribe absolute values to either organ competence or hormone concentration but rather that we have to measure one in terms of the other.

We have now to consider in more detail certain aspects of the problem of hormone-controlled growth and differentiation which have arisen in the course of these investigations. For this it seems best to discuss separately the principal points in question, and after we have estimated their value to try to fit them into the framework of the general concept.

A. Relationship between hormone concentration and effective level.

Of particular interest is the observation that organ discs transplanted into adult female hosts are able to grow even in the absence of ring glands. Since we know that the growth of the transplanted organ is under the control of the ring gland hormone we might assume that female hosts, in contrast to male hosts, either produce or have stored some hormone. We know further that two ring glands have the same effect as four glands. In the presence of two or four ring glands, therefore, the environment of either female or male hosts must be considered saturated with hormone as far as the growth of the organ is concerned. We should thus expect the hormonal environment of female and male hosts to be the same, i.e. saturated when both hosts are supplied with five ring glands each. Consequently, we should also expect the growth response of identical organs grown in such a saturated male and female environment to be the same. This, however, is not the case, as the experiments show (see p. 42). The discs in the female environment grow much better than their partners in the male environment, although both were in a hormone-saturated environment. This suggests that the ring gland hormone does not act directly but, rather, indirectly by the intervention of some factors in the host. Limited by the lack of further knowledge on this point we might assume for the time being that the ring gland hormone establishes what might be called an "effective level" in the host, which in turn is responsible for the various reactions of the test organs. This assumption is supported by the fact that we observe similar differences in the reaction of the test organs under the influence of the same number of ring glands in different species. In these cases, too, a different growth effect is produced when the hormone concentration has saturated the environment.

For example, we find that two ring glands in melanogaster hosts have a greater effect on the growth of the test organ than four ring glands have in virilis hosts. However, there is a definite relationship between the hormone concentration and the effectiveness of this level. We find that a low hormone concentration produced by one ring gland is unable to raise the host level to its most effective state, while the hormone concentration produced by two ring glands already brings the level to its highest state of effectiveness. Although the effective level cannot be raised above a certain threshold even when higher hormone concentrations, i.e. more ring glands, are used, its peak effectiveness is nevertheless higher in females than in males and in melanogaster than in virilis. Yet there is no apparent difference in the effective level of the females in these two species when tested without ring glands. The difference between the species becomes evident only when their levels are elevated by the ring gland hormone. Whether the low effective level of female hosts is caused by the presence of a small amount of hormone is as yet still obscure, but of course possible. In any event, it seems unlikely that some hormone is stored, since in this case we would expect that the stored hormone would gradually decrease as the flies become older. The experiments show, however, that young and old flies are equally affected. Now, when one follows the thread of implications connecting these various points it becomes evident that one may obtain different effective host levels either by varying up to a certain point the hormone concentration, or by varying the host animals. For example, the lowest effective level prevails in female hosts without ring glands. The effective level is somewhat higher in male hosts with one ring gland. In melanogaster and virilis male hosts with two or more ring glands, the effective level is lower than in the virilis female hosts with two or more glands, while in a melanogaster female with two or more ring glands the effective level is highest. If in the following we speak of hormone concentration, it should be understood that we always refer to a host level of a certain effectiveness, produced by a definite concentration of ring gland hormone in a definite host.

It is characteristic that organ discs are unable to grow in adult male hosts without the support of ring glands. The male host environment was thus considered neutral. Now we know only that the adult male environment is neutral as far as the larval discs are concerned. Whether pupal organs which are presumably much more responsive than larval organs are also unable to develop in male hosts is not known so far. Actually it would be very difficult to prove that such an environment is neutral in an absolute sense, i.e. for all larval as well as all pupal tissues. If we should find, for example, that pupal discs, but not larval discs, would develop in adult male hosts and from this conclude that the pupal discs have attained the capacity of independent development, this conclusion could well be erroneous. We must take into consideration that the effective level in the male hosts, although too low for the growth of the larval discs, might well be high enough to assure the development of the highly responsive pupal discs. This argumentation brings us directly to one further aspect of the problem. In an earlier paper (Bodenstein, 1939a) it was shown that eye discs of young pupae continue their development when transplanted into larvae the anterior parts of which were cut off by means of a ligature. From these experiments the conclusion was drawn that pupal eye discs, which already had been stimulated by the differentiation-promoting hormone, are able to develop independently in an environment lacking the differentiation stimulus. At the time these experiments were performed we did not know that the ring gland is the source of the hormone which promotes differentiation, nor that this hormone is produced in younger larval stages. The larval host therefore was expected to contain no differentiation hormone. Although the source of the hormone supply in these earlier experiments was cut off by the ligature, and thus no hormone coming from the ring gland could have reached the transplant, it is highly probable that enough hormone was left in the rear part to account for the continued development of the transplanted organ. Since we must assume that even a very low hormone concentration is sufficient to affect the very responsive older eye discs, this experiment does not prove the independent development of the pupal eye. Ephrussi (1943) has recently performed a similar experiment. He transplanted eye discs from mature larvae into the abdomen of young larvae and observed that these discs developed synchronously with the host organs. However, in another series of experiments where he transplanted eye discs of one-day old pupae into young larvae he found the transplanted eyes to develop heterochronously. In these cases the transplanted pupal eye had already formed red pigment while the hosts were still in their larval stage. These experiments also do not prove the independence of eye development, for the hormone concentration in the young larvae, although not high enough for the differentiation of the larval host organs, might have been sufficient for the differentiation of the pupal eye. In the light of these considerations, it is very difficult indeed to be sure whether one is dealing with dependent or independent development. Again we are confronted with the fact that development is not the reflection of absolute conditions, but is highly relative indeed; it is the expression of a very delicate balance between the activating and reacting systems involved.

B. The effective level and tissue competence.

It takes about eight days for a leg disc to differentiate to imaginal completion in a very effective female environment obtained by a hormone concentration produced by two or more ring glands, while in normal development in the presence of only one ring gland the leg disc completes its differentiation in four days. This shows that the effective level in the normal pupal environment must be much higher than that of the most effective adult environment. This low level in the adult environment is very fortunate for the understanding of the responsive capacity of the test organs, since it has brought to light real differences in the responsiveness of different test organs and of different regions within identical organs. For example, if we compare different discs as to their capacity to differentiate, we find in the most effective adult environment only the distal parts of leg discs are able to complete their imaginal differentiation, while under the same conditions, eye discs differentiate only partially and genital discs not at all. These differences in the responsive capacity of the different discs are not detectable if we grow them in a pupal environment under the influence of a very effective level. For, if we transplant legs, eyes and genital discs into larvae shortly before pupation, all these discs become mature in complete synchrony with the host organs and there seems to be no difference between them as far as their responsiveness is concerned. We have demonstrated that young and old discs grown in a highly effective adult environment differ in their time of onset of differentiation. The young leg discs begin and complete their differentiation considerably later than older leg discs. When finally even the young discs have attained imaginal character they are of approximately the same size as the older discs. In other words, the young discs grow to a certain size before their differentiation leading to imaginal completion begins. This seems in disagreement with the results of earlier experiments (Bodenstein, 1939b) where it was found that young eye discs transplanted into older larval hosts differentiated prematurely, that is, before they had reached their full larval size, and as a consequence were finally smaller than normal eyes. When we recall that the effective pupal level is much higher than even the most effective level in an adult environment, we realize how we can explain the observed discrepancies between the results of our earlier and present experiments. Obviously, the pupal level is high enough to induce premature differentiation in the young organ while the adult level is able only to promote growth in the young organ. Only after the young disc in the adult environment has grown to a certain stage and has thereby become more readily responsive is the low effective adult level able to induce differentiation also into the young disc. Experiments in which the responsiveness of young and old salivary glands was tested (Bodenstein, 1943a in press) yield the same results. These experiments show that differentiation takes place only when both organ-responsiveness and effective level together attain a sufficient value. The difference between the responsive capacity of young and old organ discs is also clearly demonstrated by experiments (Bodenstein, 1939a and b) in which very young eye discs were transplanted into larvae shortly before pupation. In these cases the very young eye discs were only partly differentiated at the time the host emerged, although they had been under the influence of the very effective pupal level. This shows that even the very effective pupal environment is unable to bring about complete differentiation in test organs which are very young and hence possess a very low responsive value.

If we list the different organ discs as to their capacity to differentiate in the most effective adult environment, we find them arranged in the following order: legs, larval salivary glands, eyes, adult salivary glands and genital discs. Under the influence of the same effective adult level we thus find that the value for the differentiation response is highest in the leg disc and lowest in the genital and adult salivary gland discs, while the values for the other discs tested fall between these extremes. However it seems that the larval skin is more readily responsive than all the organ discs, as the following experiments by Hadorn and Neel (1938) indicate. The authors transplanted ring glands into young larvae of the early third instar and found that under the influence of the ring gland grafts puparium formation took place prematurely, yet these prepupae failed to develop further. This indicates that the larval skin is very responsive indeed, since it responded to the increased hormone level with puparium formation, before the organ discs were able to respond and hence failed in their differentiation.

Viewing the specific results of the investigations we conceive the following general picture of the mode of action of the ring gland in the development of Drosophila. The larval ring gland of Drosophila is an organ of internal secretion which produces its hormone during the entire larval period. This hormone controls the growth of organ discs during larval life. In the course of larval development the ring gland becomes larger and produces more hormone, while at the same time the responsiveness of the organ discs increases as they grow older. When the hormone concentration and the responsiveness of the organ discs have reached a certain value, the ring gland hormone controls imaginal differentiation also. The evagination of the organ discs is the first indication that they have reached a differentiation phase. In normal development this stage is reached at the time of pupation. Pupation is thus nothing more than the first step in the process of differentiation. The kind of organ response, i.e. whether the organ discs respond with growth or differentiation to the ring gland hormone depends upon a definite relationship between hormone concentration and organ responsiveness. It is very probable that the ring gland hormone has no direct effect on the reacting organ systems, but that it rather acts indirectly through the intervention of some as yet unknown mechanism. If these conclusions deduced from experimental results are correct, it should follow that extirpation of the ring gland in the larval stage should prevent the growth of the organ anlagen. This experiment, technically not possible in Drosophila, has actually been performed by Burtt (1938) on Calliphora larvae, with the result that the growth of the organ disc was arrested in larvae which had their ring glands removed. These experiments then provide further evidence that the ring gland hormone controls not only differentiation but also the processes of organ growth during the larval period. The general interpretation of the problem under discussion is in contrast to Hadorn's view; he maintained that only ring glands from mature larvae produce hormone and that this hormone controls solely the processes of puparium formation, but has no effect on the growth or differentiation of the organ discs. On the basis of our experimental evidence, Hadorn's conception seems to be no longer tenable.

SUMMARY

A variety of organ discs of Drosophila was transplanted together with or without ring glands into the body cavity of adult flies and their developmental behavior in their new surroundings studied. The specific results of these investigations are briefly summarized as follows:

1. Organ discs transplanted into adult male hosts cease to develop but remain alive presumably indefinitely. The transplanted discs do not lose their developmental potencies, although development may be arrested for a long time.
2. Organ discs transplanted into adult male hosts will grow and finally differentiate to imaginal completion when under the influence of simultaneously transplanted ring glands.
3. Organ discs transplanted into adult female hosts continue their development at a very slow rate even in the absence of ring glands.
4. There is no difference in the organic environment of different species as far as the development of test organs is concerned. If however, different host species are provided with the same number of ring glands it is found that the ring glands have a greater effect on the development of the test organs in melanogaster than in virilis hosts.
5. Ring glands of all larval ages, even from larvae only 12 hours old, are able to induce growth in the transplanted test organ.

6. The amount of hormone produced by young larval ring glands is less than that produced during the same time interval by ring glands of mature larvae.

7. Different organ discs differ as to their capacity to differentiate in adult hosts under the influence of ring glands.

8. Different regions within the same organ disc also differ as to their differentiation capacity.

9. Under the same hormonal environment it takes the young organ discs a considerably longer time to complete differentiation than it takes the old organ discs.

10. The ring gland hormone, apparently, does not affect the reacting organ directly, but acts rather through the intervention of some as yet unknown factors in the host.

11. The kind of organ response, that is, whether the organ disc responds with growth or differentiation to the ring gland hormone depends upon the relationship between hormone concentration and organ responsiveness.

12. The problem of growth and differentiation in the development of Drosophila is discussed. It is pointed out that development is not the reflection of absolute conditions but that it is highly relative indeed; it is the expression of a very delicate balance between the activating and reacting systems involved.

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THE LIFE–HISTORY OF *PHYLLODISTOMUM SOLIDUM* RANKIN, 1937, WITH OBSERVATIONS ON THE MORPHOLOGY, DEVELOPMENT AND TAXONOMY OF THE GORGODERINAE (TREMATODA)[1]

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Introduction

Digenetic trematodes are internal flatworm parasites which occur in all classes of vertebrates. The life-cycles of these parasites involve two or more hosts of which one, the first intermediate, harbors the parasitic asexual stages and eventually liberates a transfer stage which is typically a free-swimming cercaria; the definitive host is usually a vertebrate which harbors the parasitic sexual stages of the worm. The cyclic transfer from one host to another has been variously modified by interpolations of additional intermediate hosts, and by complications in the methods of infection of the first intermediate hosts.

Trematodes belonging to the family Gorgoderidae Looss, 1901 are characterized by having their bodies generally divided into two regions: a narrower, mobile preacetabular part and a broader, sluggish postacetabular region. The cuticula is usually smooth. The intestine may be simple to ramified. The genital pore is median, preacetabular and behind the bifurcation of the gut caeca. The testes, two to nine in number, are intercaecal or extracaecal, usually oblique, rarely opposite. The ovary is usually pretesticular. The vitellaria are paired and postacetabular. The uterus is much coiled postacetabularly. The eggs are relatively small, numerous, without filaments, and usually fully embryonated. Adults are found in the excretory ducts and urinary bladders of fishes, amphibians and reptiles, and in the body and pericardial cavities of marine elasmobranchs.

The Gorgoderidae consists of two subfamilies with contrasting characters as follows:

Gorgoderinae Looss, 1899	Anaporrhutinae Looss, 1901
1. No pharynx.	1. Well-developed muscular pharynx.
2. Laurer's canal present.	2. No Laurer's canal.
3. No seminal receptacle.	3. Prominent seminal receptacle.
4. Parasites in urinary bladders and ducts of fishes, amphibians and reptiles.	4. Parasites in pericardial and body cavities of marine elasmobranchs and urinary bladders of marine turtles.

The subfamily Gorgoderinae, at present, contains the following genera: *Gorgodera* Looss, 1899; *Gorgoderina* Looss, 1902; *Phyllodistomum* Braun, 1899; *Xystretum* Linton, 1910; *Macia* Travassos, 1922. The genus *Phyllodistomum* has synonymy as follows: *Spathidium* Looss, 1899; *Catoptroides* Odhner, 1902; *Microlecithus* Ozaki, 1926; *Dendrorchis* Travassos, 1926.

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The subfamily Anaporrhutinae, according to Nagaty (1930), contains the following genera: *Anaporrhutum* Ofenheim, 1900; *Probolitrema* Looss, 1901; *Plesiochorus* Looss, 1901; *Petalodistomum* Johnston, 1912; *Staphylorchis* Travassos, 1920.

Generic diagnosis of *Phyllodistomum* Braun: Body usually spatulate. Ventral sucker usually larger than oral sucker. Intestinal crura separate posteriorly. Genital atrium usually present. Testes two, smooth to deeply lobed, usually oblique, the one on ovarian side being more posterior. Anterior testis may be cephalad of ovary. Vas deferens long, seminal vesicle conspicuous, pars prostatica and ductus ejaculatorius generally short and inconspicuous. Ovary entire or lobed, usually posterior or lateral, rarely anterior to vitellaria. Oviduct relatively long, arising from dorsum of ovary. Fertilization space usually evident. Laurer's canal usually paralleling vitelline duct on side opposite to ovary and opening to exterior. Mehlis' gland present but indistinct. Metraterm large and distinct. Vitellaria compact or lobed; common vitelline duct very short. Eggs in metraterm embryonated. Excretory vesicle sac-like, elongate, its pore usually subterminal.

Gorgoderid trematodes are usually found in the exretory systems of their hosts; however, von Olsson (1876) described *Distoma conostomum* from the esophagus and gills of *Coregonus maraena*. Nybelin (1926) stated that the worms probably had emigrated from the bladder upon the death of the host, and that he always found this species of trematode in the urinary bladder. Van Cleave and Mueller (1934) have likewise reported ectoptic *Phyllodistomum superbum* from the gut of *Esox lucius*, *Percina caprodes zebra* and *Percopsis omnisco-maycus* from North America. Both Looss (1899) and Odhner (1902), upon the basis of morphological similarity with other known phyllodistomes, agreed that von Olsson's *Distoma conostomum* is a species of *Phyllodistomum*.

There have been more than 40 seemingly valid species described in this genus.

According to Nybelin (1926) the first report of an adult phyllodistome is probably that of Fabricus (1780) who described *Fasciola umblae* from the "kidney" of *Salmo alpinus* ("*in sanguine dorsali salmoni alpine*"). Later Fabricus (1794) redescribed and figured this same form. Rudolphi (1819) renamed it *Distoma seriale,* expressing the opinion that "*sanguine dorsali*" is probably to be interpreted as the kidney.

In the year 1816 v. Olfers described from *Esox lucius* a bladder fluke which he called *Distomum folium*. Many of the distomes found by subsequent workers in the bladders and urinary ducts of fishes and amphibians have been referred to this species. This fact has created the extremely difficult taxonomic problem of attempting, with meager data, the separation and identification of valid species.

Braun (1899) erected the genus *Phyllodistomum* with *Dist. folium* Olfers, 1816, as type, and included in the genus: *Dist. cygnoides* (Zed.) Looss, *D. cymbiforme* Rudolphi and *D. patellare* Sturges. Later in the same year Looss erected the genus *Spathidium* with *D. folium* Olfers as type. By the laws of taxonomic priority, *Spathidium* has been suppressed as a synonym of *Phyllodistomum*.

Zschokke (1884) found *Distomum folium* in *Cottus gobio*, *Thymallus vulgaris*, *Trutta variabilis* and *Salmo umbla*, but not in *Esox lucius*. Braun (1892) found

Distomum folium in *Esox lucius* at Königsberg. His account indicates that Zschokke had confused: the ovaries (2) with the vitellaria, the oviducts with the vitelline ducts; he failed to see the ovary; the shell gland was confused with the vitelline reservoir and the vitellaria were eggs in the uterus. Looss (1894) reported *Distomum folium* from the urinary ducts of *Acerina cernua*. Sinitsin (1905) studied fishes from Warsaw ponds and identified *Phyllodistomum folium* in *Carassius vulgaris*, *Barbus vulgaris*, *Gobio fluviatilis*, *Leuciscus rutilus*, *Scardinius erythrophthalmus*, *Squalius cephalus*, *Idus melanotus*, *Aspius rapax*, *Abramis brama* and *Blicca björkna*. Finally, Zandt (1924) found *Phyllodistomum folium* in *Leuciscus leuciscus* and *Leuciscus rutilus* from Lake Constance.

Looss (1901) described *Phyllodistomum acceptum* from *Crenilabrus pavo* and *C. griseus*. Odhner (1902) described *Phyllodistomum unicum* from *Serranus sp.*, *Phyllodistomum linguale* from *Gymnarchus niloticus*, *P. spatula* from *Bagrus docmac* and *B. bayad*, and *P. spatulaeforme* from *Malapterurus electricus*. He hesitated in placing the last two phyllodistomes in the genus because they had symmetrically placed testes and sharply separated anterior and posterior body regions. It was due to this hesitation that Odhner in Looss (1902) erected the questionable genus *Catoptroides* with *P. spatula* Odhner as type.

Osborn (1903) described *P. americanum* from North American *Amblystoma tigrinum*. Since that time, descriptions of phyllodistome species have been generally complete enough to reduce taxonomic difficulties.

Two complete and several incomplete life-histories have been reported for members of this subfamily. Incomplete cycles were determined by Sinitsin (1905) for three species of *Gorgodera* and *Gorgoderina vitelliloba*. Krull (1935) experimentally proved the life-cycle of *Gorgodera amplicava*. Rankin (1939) determined the life-history of *Gorgoderina attenuata*, the first for any North American species of that genus. Except for abstracts by Crawford (1939, 1940) on the life-history of *Phyllodistomum americanum*, and Goodchild (1940) on the life-history of *P. solidum* there have been no life-histories reported for North American phyllodistomes.

Several European workers have postulated, upon morphological similarity and slight experimental evidence, certain relationships between cercariae or metacercariae and sexually mature phyllodistomes. Nybelin (1926) summarized and amplified these speculations concerning European phyllodistome life-histories. Looss (1894), Lühe (1909) and Odhner (1911) proposed that *Cercaria duplicata* v. Baer which develops in *Anodontites cygnea*, *Anodontites anatina* and *A. cygnea ventricosa* is the larva of *Phyllodistomum folium* (Olfers). Nybelin (1926), on the other hand, stated his belief that *C. duplicata*, as at present defined, consists of three separate cercariae: (1) *C. duplicata* Wagener, 1851 (Nybelin stated that this larva must be removed from the phyllodistome cercariae because it possesses a pharynx and has a Y-shaped excretory bladder); (2) *C. duplicata* Pagenstecher, 1857 (also accredited with a pharynx, which Nybelin regards as an error in observation)—Nybelin was unable to link this cercaria with any known adult phyllodistome; (3) *C. duplicata* Reuss, 1903 (= *C. duplicata* v. Baer according to Nybelin). In extensive feeding experiments of metacercariae from *C. duplicata*, using 16 different species of fish, several of which are natural hosts for *Phyllodistomum folium*, Reuss was unable to obtain any sexually mature distomes. He found metacercarial excystment only in two species, *Cyprinus* *carpio* and *Tinca vulgaris*, but there was no progressive development; thus he was unable to determine the species of the adult phyllodistome. Nybelin postulated, upon the basis of the sucker ratio, the position of the genital primordium and the position of the genital pore, that *C. duplicata* of Reuss is the larva of *Phyllodistomum elongatum;* this hypothesis seems untenable because Reuss was unable to find metacercarial excystment in *Abramis brama* which Nybelin listed as a normal host for the sexually mature *P. elongatum.*

Nybelin believed that the short-tailed cercaria described by Sinitsin (1901) as the larva of *Phyllodistomum folium* is a true phyllodistome cercaria, but is not that of *P. folium* because the cercaria had an acetabulum smaller than the oral sucker; the smallest adult *P. folium* which Nybelin observed (0.45 mm. long), had an acetabulum larger than the oral sucker. Nybelin stated that this short-tailed cercaria may represent the larva of *P. elongatum*, the other known bladder fluke of cyprinids. This assumption is inconsistent, however, because in the same paper he had already proposed the *C. duplicata* of Reuss to be the larva of *P. elongatum*, "Die einzige Larvenform für welche eine Vermutung der Artzugehörigkeit einigermassen berechtigt erscheint, ist die von Reuss näher behandelte, welche durch ihre etwa gleichgrossen Saugnäpfen, durch die Anlage des vorderen Hodens schräg hinter der Anlage des Germariums und vor allen durch die dicht vor dem Bauchsaugnapf gelegene Anlage des Genitalporus sehr an *Ph. elongatum* erinnert." This seeming contradiction Nybelin avoided by a second assumption as to the fate of the cercaria described by Reuss, "Es ist also auch in diesem Falle nicht möglich, etwas bestimmtes zu sagen; es wäre sogar denkbar, dass sich Reuss' Cercarie zu *Ph. pseudofolium* entwickeln könnte." However, the validity of this species has been questioned; Lewis (1935) threw *P. pseudofolium* into synonymy with *P. folium.*

Odhner (1911) stated that the stumpy-tailed cercaria of Sinitsin (1901) is the larva of *P. macrocotyle.* However, Nybelin (1926), Zandt (1924) and Lewis (1935) agreed that *P. macrocotyle* is synonymous with *P. folium.* Odhner (1911) believed that the marine "Rattenkönigcercaria" *Cercaria clausii* when eaten by the fish *Chrysophrys aurata* develops into *Phyllodistomum acceptum.* This belief was accepted by Steelman (1938) who accordingly suppressed *P. acceptum* as a synonym of *Phyllodistomum clausii.*[3] Nybelin summarized these life-history studies and speculations in his statement, "Es ist also gegenwärtig für keine *Phyllodistomum*-Art möglich, die zugehörige Cercarienform sicher anzugeben; die postembryonale Entwicklungsgeschichte der einzelnen Arten dieser Gattung muss vielmehr ganz von neuem, und zwar am bestem auf experimentellem Wege studiert werden." This is the only way reliable results are obtainable; and furthermore, life-history studies based upon controlled experimentation will be invaluable for clarification of the chaotic taxonomic status of the sexually mature phyllodistomes.

The first phyllodistome life-history to be experimentally completed in the laboratory was reported by Goodchild (1940) for *Phyllodistomum solidum* Rankin, 1937 which consisted of the following stages: sexually mature distomes in the urinary bladder of *Desmognathus fuscus fuscus*, sporocysts and young cercariae in *Pisidium abditum*, and metacercariae in naiads of several species of Odonata.

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[3] Cable [1942. *Jour. Parasit.* 28 (6 suppl.)] proposes that *Cercaria clausii* is the larva of a fish trematode belonging to the family Lepocreadiidae or the family Gyliauchenidae.

In the present paper, the various stages of the parasite are described and experiments proving the life-cycle are cited.

The author wishes to acknowledge the encouragement and helpful criticism given by Professor H. W. Stunkard during both the progress of the experimental portions of the study and the preparation of the manuscript.

Materials and Methods

A. Bivalves and asexual stages.

The fresh-water sphaeriid hosts of gorgoderid trematode asexual larval stages are grouped into the three genera: *Sphaerium*, *Musculium* and *Pisidium*. *Pisidium abditum*, host of *C. Phyllodistomum solidum*, were collected from Barrett Pond near Cold Spring on Hudson, N. Y., in October and November 1937, and during the summers of 1938, 1939 and 1940. The clams were collected by careful examination of small quantities of bottom in regions where they were abundant. In this way, two collectors were able to take about 300 clams in one day. In the laboratory they were separated into lots of about 25 to 30 each and placed with a small amount of pond debris into finger-bowls. They were then set in running water to maintain a lower temperature. Every morning each container was examined for cercariae with the aid of a 3X lens; this method proved adequate for locating the cercariae, but as a final check, each bowl was examined under a 17X binocular microscope.

When cercariae were found in any dish, the clams in that bowl were then isolated to obtain the infected ones. All infected bivalves were placed in individual containers; this separation reduced the death rate because infected clams are less tolerant of conditions of crowding, increased temperature, starvation and fungal infections than uninfected molluscs.

It was extremely difficult to keep the clams alive in the laboratory. The water in which they lived was changed at least once a day. Culture water containing various species of green algae and diatoms was introduced in small amounts at frequent intervals. Nevertheless, the mortality during the period of adjustment to laboratory conditions was discouragingly high. When kept in running city water the results were more encouraging. Finally, an aquarium was set up which approximated the conditions in nature as much as possible. The pond debris which served as the bottom was sloped to one end of the tank. The depth of water was approximately two inches in the deepest part. At the other end, moss was introduced and a fine stream of water was allowed to trickle over it. It was possible in this way to have laboratory-raised specimens for miracidial infection experiments.

Infected bivalves were teased apart to obtain mature living sporocysts for study. Mature sporocysts were also fixed in warm (65°) Bouin's fixative and stained with paracarmine and Delafield's haematoxylin.

B. Odonatan naiads and metacercariae.

The damsel-fly naiads which were used as the second intermediate hosts were both grown from eggs in the laboratory, and collected from a small pond in Van Cortlandt Park, New York City. This pond was free of *Pisidium sp.*, so the naiads were the equivalent of laboratory-raised specimens. As a control, 25 per cent of the damsel-flies were dissected under the binocular microscope; they were all free of any helminth metacercarial infection. In addition, the naiads which were to be used as hosts were isolated in the laboratory for two weeks, after which time they were starved for periods of from three to seven days. Starvation made them so transparent that examination by transmitted or reflected light would disclose immediately any metacercariae. This period of starvation served the additional function of causing the naiad to capture and to devour eagerly any cercariae offered.

The cercariae were removed singly by micropipettes and introduced into a finger-bowl with a starved damsel-fly naiad. As soon as the nymph had seized a cercaria it was placed under the microscope and the penetration, wandering and encystment of the cercaria were watched.

Encysted and mechanically excysted metacercariae were studied alive, and also after fixation in Bouin's fluid and staining with Delafield's haematoxylin, Ehrlich's haematoxylin and paracarmine. Serial sections of experimentally infected odonatan naiads were also studied.

C. *Desmognathus fuscus fuscus and adult worms.*

The salamanders which were used in the experiments were captured as young adults. They were kept in the laboratory for periods of from 12 to 17 months before being used. During this time they were isolated at monthly intervals for periods of from three to seven days in a small quantity of water in a finger-bowl. The water in which they were living was examined about four times a day for free-swimming miracidia. Since, in agreement with Rankin (1939), it was found that miracidia will live under these conditions for as long as 24 hours, any miracidia shed would be readily found. Salamanders kept under identical conditions as controls, when dissected, were always found to be parasite free.

Adult worms were studied alive and after fixation. Bouin's fluid and saturated mercuric chloride solution with about 5 per cent acetic acid added were used as fixatives. Whole mounts were stained with paracarmine. Serial sections were stained with Ehrlich's haematoxylin and Mallory's triple connective-tissue stain.

EXPERIMENTS

1. Experiments with first intermediate hosts:

Infected *Desmognathus fuscus fuscus* were isolated in finger-bowls in small quantities of water. The miracidia, which were given off in great numbers, were picked up in a dropper and introduced into a finger-bowl containing actively crawling laboratory-raised bivalves which were about 2 mm. long and approximately six months old. The miracidia moved about in more or less direct courses. They were not attracted to the bivalves; they would swim within less than 0.1 mm. of the clams and not change their courses. Miracidia were observed being drawn into the mantle cavity of the bivalve by way of the incurrent mantle cleft. The miracidium of *Gorgoderina attenuata*, according to Rankin (1939), enters its bivalve host similarly.

A more successful method of infection of molluscs was to introduce an infected *Desmognathus* into an aquarium with clams enclosed in small wire baskets. The salamander and bivalves were then allowed to remain together for as long as one week. It was possible, in this way, to obtain 50 to 70 per cent infections in the clams.

2. Experiments with second intermediate hosts:

Damsel-fly naiads were offered cercariae within four hours of emergence of the latter from bivalves. The violent action of the cercariae attracted the attention of the insect nymph. The labium of the naiad was projected out to grasp the cercaria which was then pulled within reach of the mandibles. The enlarged portion of the tail, because of its turgid spherical cells, prevented damage to the distome in the chewing process. However, it was found that such protection is not absolute, because about 15 per cent of the cercariae eaten failed to establish themselves in the insect host. Some of the failure may be due to imperfect cercariae, but in other cases, lacerated and partly digested cercariae have been found in sections of the gut.

The insect nymph was able to engulf the cercaria completely in approximately ten seconds. The tail collapsed and was digested, while the young distome penetrated the intestinal wall. Usually only one minute elapsed from the time the damsel-fly naiad took the cercaria until the young distome had penetrated the crop wall. In one instance, the point of penetration was in the prothorax; the worm migrated posteriad to the anterior edge of the metathorax, then turned and wandered to the anterior edge of the mesothorax where encystment finally took place. Usually the cercaria encysted, within four minutes of penetration, in the segment in which it pierced the crop.

The act of penetration caused apparent discomfort to the insect. With one cercaria the reactions were as follows: within 15 seconds after the act of swallowing, the naiad rubbed the legs together and also rubbed the body with the legs; then it started nervous wriggling movements, followed by short random dashes through the water. If several cercariae were taken within ten seconds of each other multiple penetrations occurred. The initial symptoms of the insect were then intensified, but otherwise similar to those described above. However, after about one minute the naiad lost its equilibrium and went into tetanus. A damsel-fly thus treated would not take additional cercariae for at least 30 minutes.

Similar behavior of odonatan naiads during penetration of gorgoderid cercariae has been reported by Sinitsin (1905) and Krull (1935).

The forming metacercarial cyst wall was recognizable as a delicate flexible covering within 15 minutes after cercarial penetration. The anterior end of the worm could easily push the wall out of shape. After cystogenous material, which was emitted through the excretory pore, had been deposited in one region of the forming cyst, the cercarial body was then thrown into a figure-8 shape which brought the oral sucker into contact with the extruded cystogenous material. The oral sucker and edge of the mouth opening then pushed the material into a smooth layer on the inside of the growing cyst wall. Meanwhile, the posterior end of the worm had been depositing cystogenous material elsewhere. The formation of the complete cyst took approximately 18 minutes; the activity then gradually diminished, and the young worm was practically quiescent one hour after penetration. The metacercaria changed position in the cyst, as indicated by drawings made over a period of several days.

Metacercariae were usually located in the thorax of the naiads; they have been found as far posteriorly as the second abdominal segment, and as far anteriorly as the dorsum of the head.

Various odonatan species have been tried as second intermediate hosts: *Ischnura verticalis*, and *Argia sp.*, were satisfactory; *Enallagma sp.*, and *Libellula* *sp.*, were less satisfactory. *Ischnura verticalis* was easiest to obtain in numbers, and since it was the most transparent of the odonatans available, it was used extensively as a metacercarial host.

3. Experiments with the definitive vertebrate host:

Metacercariae which had been watched during cyst formation were dissected from the damsel-fly naiads and fed to *Desmognathus fuscus fuscus.* The salamanders became infected with phyllodistome bladder flukes. The metacercariae excysted in the small intestine of the urodele; young worms were recovered from the posterior portion of the large intestine, the cloaca, and the urinary bladder within 24 hours after ingestion of the metacercariae.

In one experiment, a salamander was fed, at 5 P.M., one *Ischnura* naiad containing five metacercariae; at 10 P.M., the same urodele was fed another damsel-fly nymph containing two metacercariae. The next day, at 4 P.M., the *Desmognathus* was sacrificed. Three small excysted phyllodistomes were recovered from it; one worm was found in the posterior portion of the large intestine, one in the cloaca, and one in the "urethra" migrating into the urinary bladder. All these young worms still had concretions in their excretory bladders. In routine examination of the digestive system the stomach was opened. The damsel-fly which had been eaten at 10 P.M. was still being digested; one metacercaria still encysted in it was found and studied. The stylet was attached externally to a single-layered hyaline cyst wall. This fact seems to suggest the presence of an acid-pepsin labile outer cystogenous layer. The three missing young worms were not found, although the intestine, the Wolffian ducts, the mesonephroi and the coelom were examined carefully.

Worms, of increasing size, have been recovered from the urinary bladders of the salamanders 1, 2, 3, 4, 5, 15, 21, 30, 66 and 123 days after being fed metacercariae. See Figures 9–14 inclusive.

Various vertebrates have been used in experiments with these metacercariae: *Triturus viridescens viridescens*, *Rana pipiens*, *R. palustris*, *R. catesbeiana*, *R. clamitans*, *Micropterus dolomieu*, *Eupomotis gibbosus*, *Carassius auratus* and *Cyprinus sp.*, all without success.

DESCRIPTION OF STAGES IN THE LIFE-CYCLE

1. Miracidium.

The ripe eggs in the metraterm of the uterus contain fully developed motile miracidia. As many as 300 free-swimming miracidia have been recovered from undiluted urine squeezed from the bladder of an infected salamander. When the sexually mature distome is placed in water, eggs are passed through the genital pore and hatch in 10 to 30 seconds. Ripe eggs of members of this subfamily usually hatch immediately in water, although Crawford (1940), for *Phyllodistomum sp.*, reported a two-day interval between egg deposition and

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PLATE I

FIGURE 1. Free-swimming miracidium.
FIGURE 2. Miracidium.
FIGURE 3. Miracidial epidermal plates.
FIGURE 4. Mother sporocyst (48 hours old).
FIGURE 5. Mother sporocyst.

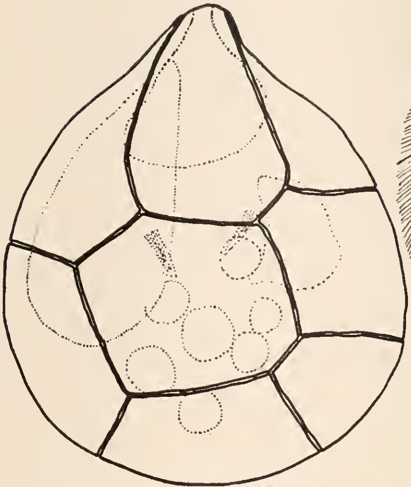

FIG. 3

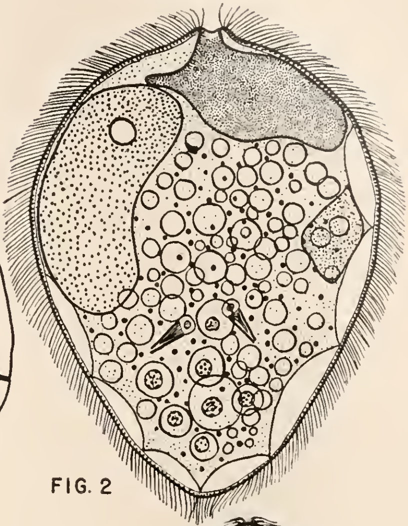

FIG. 2

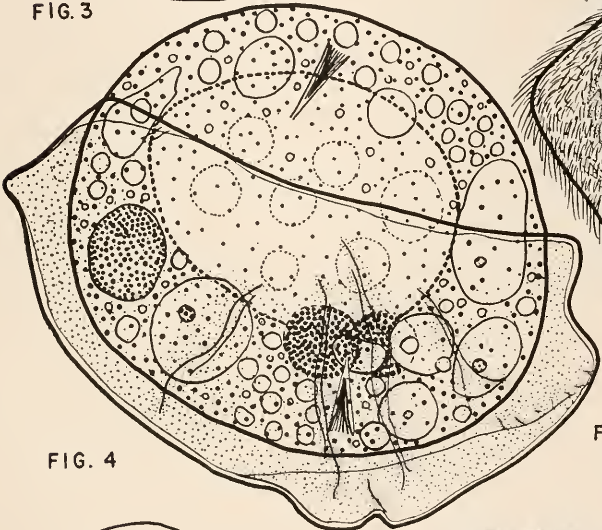

FIG. 4

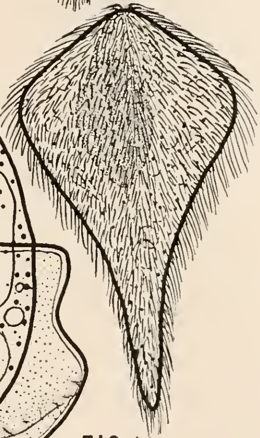

FIG. 1

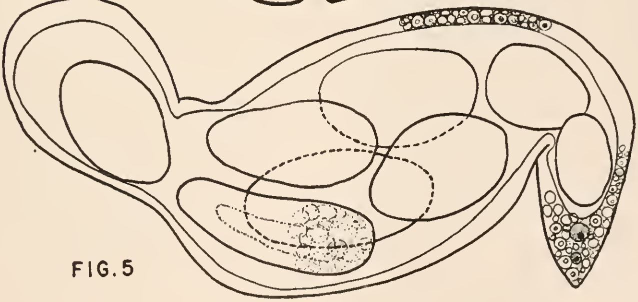

FIG. 5

PLATE 1

hatching. The free-swimming larva (Figure 1), 45 to 55 μ long by 40 to 47 μ wide, is usually mucronate-cuneate in shape, but may change to pyriform (Figure 2), often with the narrower end anterior (Figure 3). These latter shapes (Figures 2 and 3) are usually assumed when the larva is fixed or imprisoned under a cover glass.

Externally the miracidium is covered with cilia borne on 16 epidermal plates (Figure 3) arranged in three transverse rows. There are six plates in the first row, six in the second row, and four plates in the last row. Sinitsin (1905, pl. III, Figures 56 and 57) likewise indicated three transverse rows of epidermal plates in the miracidium of *P. folium*. Each cilium possesses a distinct basal granule.

Internally the miracidium is provided with an anterior sac-like "gut" 15 μ long by 24 μ wide, with a small apical opening. Situated laterally is one large gland, 34 μ long by 15 μ wide, which opens beside the "mouth." This gland is filled with refractive particles which stain deep maroon with neutral red. Rotation upon the long axis is readily observed in swimming miracidia because the asymmetrical gland appears as a rotating large, white mass. A much smaller gland, which may represent the homologue of the larger gland, is located on the opposite side of the miracidium and is often connected to the larger gland-cell by a short narrow isthmus. The body of the larva is filled with many fat-like droplets of various sizes. Similar droplets appear in all larval stages of this bladder fluke. A group of small cells with large nuclei, which may represent the germinal elements, is found in the posterior end of the miracidium. Two flame-cells are present near the posterior part of the middle-third of the body. From these flame-cells excretory ducts lead away; the ducts follow a tortuous course anteriorly and laterally for a short distance and then they turn posteriad and open separately by small lateral pores in the space anterior to the last row of epidermal plates.

2. *Mother sporocysts.*

The miracidia after being drawn into the mantle cavity penetrate the gills of the clam. The epidermal plates are sloughed off in one piece (Figure 4), exposing the subepidermal layer of the miracidium, which then becomes the wall of the mother sporocyst. The development of the larvae in the laboratory was slow. Four weeks after miracidial penetration the mother sporocysts (Figure 6) are small, 240 μ by 67 μ, and tubular, but well-organized germ-balls and developing daughter sporocysts are already present. From six to ten daughter sporocysts are produced by each mother sporocyst. Flame-cells in the young mother sporocyst are located in the peripheral thickened region of the larva. The

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PLATE 2

FIGURE 6. Young daughter sporocyst.
FIGURE 7. Mature daughter sporocyst.
FIGURE 8. Metacercaria, encysted (12 hours old).
FIGURE 9. Young adult (one day).
FIGURE 10. Young adult (five days).
FIGURE 11. Young adult (21 days).

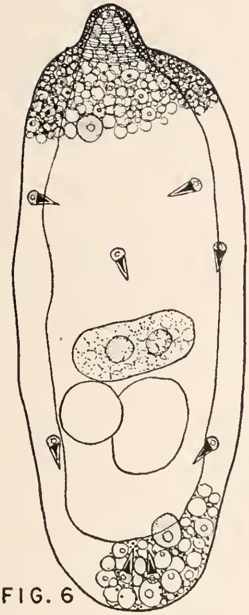

FIG. 6

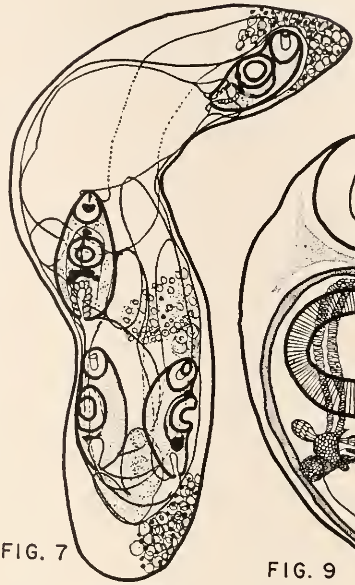

FIG. 7

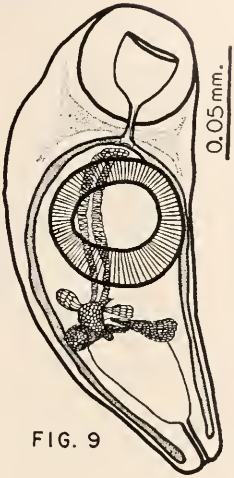

FIG. 9

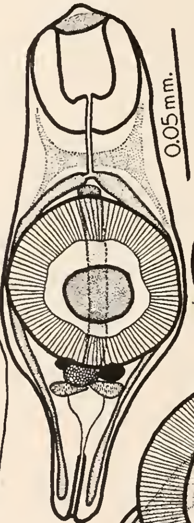

FIG. 10

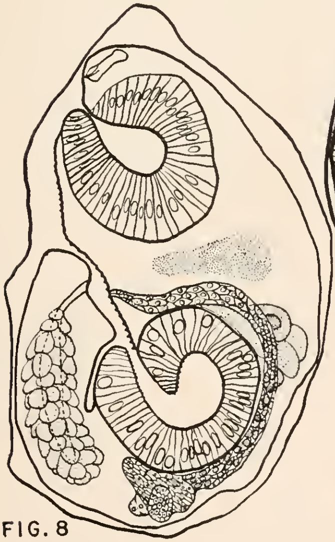

FIG. 8

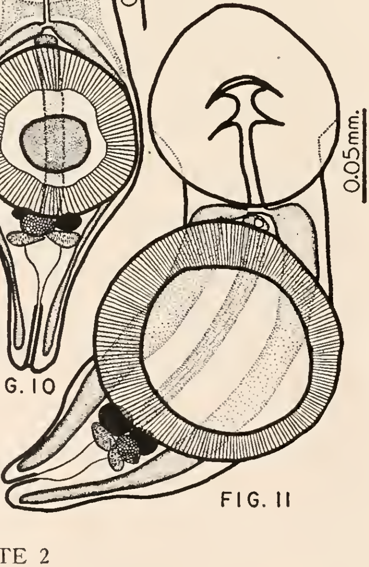

FIG. 11

PLATE 2

sporocyst wall is relatively thick (20 μ) and is composed of clear intercellular fat-like droplets and cells with large nuclei. A six-weeks old mother sporocyst measured 490 μ by 150 μ. It had eight daughter sporocysts inside which ranged in size from about 60 μ by 50 μ for the smallest, to 102 μ by 55 μ for the largest. These young daughter sporocysts already had a wall and a central cavity containing an average of three large cell groups which may be germinal in nature (Figure 5).

3. *Daughter sporocysts.*

The tubular, unbranched, sluggish, mature daughter sporocyst (Figure 7) measures about 0.67–1.25 × 0.32–0.40 mm., and lies between the outermost gill plates with its constricted, evertible anterior end embedded in the gill tissue, and its posterior free end extending into the interlamellar gill space. The sporocyst wall, 27 to 50 μ in thickness, has a thin cuticula beneath which are located very thin circular and longitudinal muscle fibers. The most conspicuous elements of the wall are clear vesicular fat-droplets ranging from 5 to 17 μ in diameter. Dobrovolny (1939) reported similar structures in *Plagioporus sinitsini* sporocysts. Definite cells of two main types also occur in the wall; the most numerous are about 10 μ in diameter with nuclei 6 to 7 μ in diameter; from two to six larger cells, 15 to 20 μ in diameter, with conspicuous granular cytoplasm are also present. Many opaque granules, about one μ in diameter, occur in the sporocyst wall. Flame-cells are similar in all respects to those described by Thiry (1859), Looss (1894), Sinitsin (1905), Krull (1935) and Vickers (1940).

The interior of the sporocyst is filled with developing cercariae. The sporocyst usually contains a number of germ-balls and a maximum of seven recognizable cercariae. The large cercarial tails occupy most of the space in the sporocyst. The developmental stages of the cercariae approximate those described by Thiry (1859) for *Cercaria macrocerca.* A birth pore is located subterminally on the constricted anterior region. Cercariae can be forced by pressure through this pore; usually, however, the opening is indiscernible.

Between the sporocysts there are often numerous, opaque "yeast-like" cells of unknown origin and function, which aid in determination of infected clams *in vivo* by reflected light before cercariae are shed. The sporocysts are usually grouped in the posterior to postero-dorsal gill regions. With reflected light they appear lighter in color; with refracted light they appear as a darker mass. Unfortunately, clams which are carrying young in the marsupium present much the same appearance with both types of illumination; however, the developing young clams are located in the center of the gill region, while the parasites are located more posteriorly.

4. *Cercaria.*

A description of the cercaria of *Phyllodistomum solidum* has already been published by Goodchild (1939a). In this same paper a discussion of the gorgoderid cercariae was given, and a review of the known species of gorgoderid cercariae with their differences from *Cercaria Phyllodistomum solidum* (= *Cercaria conica*) was also included.

Key to the Gorgoderid Cercariae

- A. Cercariae without stylets (Parasites of Unionidae and Dreissenidae)
  - B. Tail shorter than body of distome........... C. *Phyllodistomum folium* Sinitsin, 1901
  - BB. Tail longer than body of distome
    - C. Tail with an anterior enlargement and terminal thread-like portion C. *mitocerca* Miller, 1935
    - CC. Tail lacks anterior enlargement and terminal thread-like portion
      - D. European species...................... C. *duplicata* v. Baer, 1827
      - DD. American species..................... C. *duplicata* of Leidy, 1858
- AA. Cercariae with stylets (Parasites of Sphaeriidae)
  - B. Tail inactive and very elongate
    - C. Suckers of distome provided with sensory bristles; tail 4.4–7.5 mm. long C. *Gorgodera amplicava* Krull, 1935
    - CC. Suckers of distome lack sensory bristles; tail 8.8–9.6 mm. long C. *Gorgoderina attenuata* Rankin, 1939
  - BB. Tail active and shorter
    - C. With anterior chamber containing the young distome
      - D. With anterior tail enlargement
        - E. Terminal filiform portion of tail shorter than anterior swelling plus anterior chamber C. *sphaerocerca* Miller, 1935
        - EE. Terminal filiform portion of tail longer than anterior swelling plus anterior chamber
          - F. Cercarial chamber incorporated into anterior tail enlargement
            - G. Tail enlargement with lateral "wing-like" distentions C. *raicauda* Steelman, 1939
            - GG. Tail enlargement without lateral "wing-like" distentions C. *Gorgoderina vitelliloba* Sinitsin, 1905
          - FF. Cercarial chamber in front of and distinct from the tail enlargement
            - G. Anterior tail enlargement filled with spherical cells
              - H. Chamber containing distome ovoid; anterior swelling 1/10–1/13 of total tail length..................... C. *macrocerca* Thiry, 1859
              - HH. Chamber containing distome conical; anterior swelling 1/4–1/5 of total tail length.................... C. *Phyllodistomum solidum* Goodchild, 1939
            - GG. Anterior tail enlargement filled with polygonal cells
              - H. Filiform portion of tail with four equidistant compact longitudinal muscle bands..................... C. *coelocerca* Steelman, 1939
              - HH. Filiform portion of tail without compact longitudinal muscle bands
                - I. Distome with 9 pairs penetration glands; anlagen of testes in 5 parts C. *Gorgodera varsoviensis* Sinitsin, 1905
                - II. Distome with 4 pairs penetration glands; anlagen of testes in 9 parts C. *macrocerca* Wagener, 1857 according to Sinitsin (1905)
      - DD. Without anterior tail enlargement
        - E. .... C. *donecerca* Goodchild, 1939
        - EE. Undescribed....................... C. *Phyllodistomum sp.* Crawford, 1940
    - CC. Without anterior chamber.............. C. *macrocerca* Wagener, 1857 according to Wagener (1857)

The presence of stylet-bearing and non-stylet-bearing cercariae in the same trematode genus is unique. In the Gorgoderidae, moreover, certain morphological and physiological relationships always accompany the stylet condition: non-stylet gorgoderid cercariae possess precociously differentiated testes, lack both penetration glands and definitely organized cystogenous gland cells around the excretory bladder, have a postacetabularly flared body, and parasitize members of the family Unionidae and Dreissenidae; non-stylet cercariae lack intermediate hosts according to Sinitsin (1901) and Reuss (1903); the adults of these cercariae are parasites of fresh-water fish. Stylet bearing gorgoderid cercariae, on the other hand, lack precociously developed testes [except *C. Gorgodera cygnoides*, *C. Gorgodera pagenstecheri*, *C. Gorgodera varsoviensis* and *C. Gorgoderina vitelliloba* described by Sinitsin (1905)], possess penetration and cystogenous gland cells, usually lack a postacetabularly flared body, and parasitize members of the family Sphaeriidae; stylet cercariae are eaten by second intermediate vector hosts in which they encyst to form infective metacercariae; adults from stylet gorgoderid cercariae are parasites of fishes and amphibians.

All sexually mature phyllodistomes have a similar morphological pattern. Upon the basis of adult structures, it is impossible to determine the cercarial type of any adult whose life-history is unknown. If subsequent investigation proves that the adults, because of similar habitats, have converged to a common morphological form from diverse ancestry (Text Figure 1) it will be a most striking case of convergent evolution; if the present concept of close adult relationships holds, however, which seems most likely, it must be assumed then that cercarial structures in the gorgoderids, at least, are only caenogenetic features which have been modified to fit the exigencies of their varied life-histories (Text Figure 2).

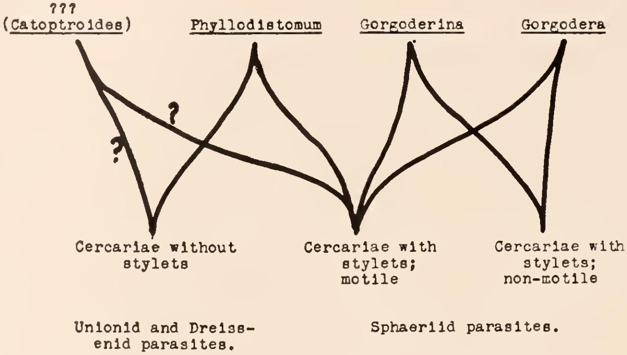
???
(Catoptroides)
Phyllodistomum
Gorgoderina
Gorgodera
?
?
Cercariae without stylets
Cercariae with stylets; motile
Cercariae with stylets; non-motile
Unionid and Dreissenid parasites.
Sphaeriid parasites.

FIGURE 1. Assuming convergent evolution of adults from diverse cercarial ancestry.

5. *Metacercaria.*

One hundred and forty-four damsel-fly naiads were each experimentally infected with from one to ten metacercariae. The latter are easily seen *in vivo* with intense reflected light because the opaque concretions in the excretory bladder appear pearly white. The metacercarial cysts (Figure 8) are spherical to ellipsoidal in shape, with the relatively inactive distome occupying practically all the internal space. The very delicate hyaline cyst wall (one to four μ thick) of cercarial origin is evidently later, approximately five days, augmented inside by a second cystogenous deposit of optically similar material. Vickers (1940) found, in *Cercaria macrocerca*, a group of about one dozen large cells with granular cytoplasm located antero-laterally to the penetration gland cells. He stated, "they may well be the true cystogenous cells." These cells may be the source of the secondary cystogenous material. Whether the cercaria of *Phyllodistomum solidum* possesses such cellular elements is unknown, however, because Vicker's staining technique was not used. The host does not aid in the formation of the cyst wall, but host cellular elements do adhere irregularly to the outer cyst membrane.

The cercarial stylet which is discarded into the cavity of the metacercarial cyst during the first 24 hours, is at first free-floating in the internal liquid; later it attached to the internal surface of the cyst and is finally embedded between primary and secondary cystogenous deposits.

The growth of the metacercaria is rapid: cysts after 24 hours measured 129 μ × 124 μ, after 15 days 162 μ × 155 μ, after 30 days 170 μ × 150 μ, after 37 days 186 μ × 150 μ and after 61 days 209 μ × 202 μ.

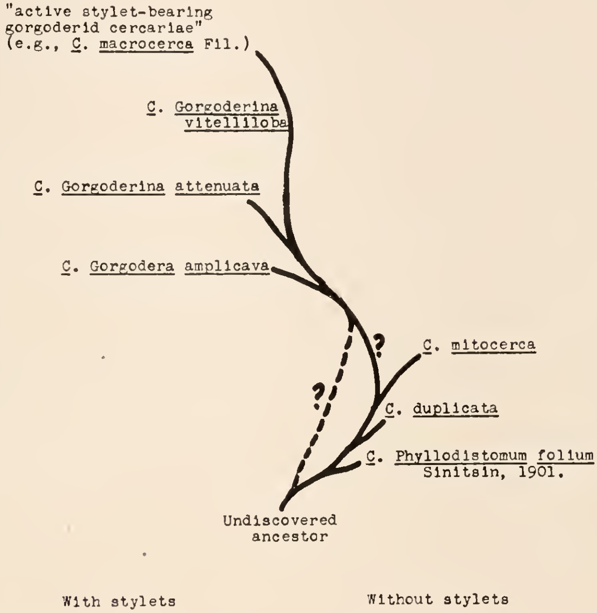
"active stylet-bearing gorgoderid cercariae" (e.g., C. macrocerca Fil.)
C. Gorgoderina vitelliloba
C. Gorgoderina attenuata
C. Gorgodera amplicava
?
C. mitocerca
?
C. duplicata
C. Phyllodistomum folium Sinitsin, 1901.
Undiscovered ancestor
With stylets
Without stylets

FIGURE 2. Assuming divergent evolution of cercariae from a common cercarial ancestry.

The worms can be excysted mechanically without damage to the young distome. The metacercaria retains the cercarial shape, but loses the oral and acetabular sensory bristles. Unlike *Gorgoderina attenuata*, as reported by Rankin (1939), the number and distribution of the sensory papillae remain constant from cercaria to adult. An anterior cavity in the oral sucker indicates the former position of the discarded stylet. The penetration glands with their ducts disappear and, unlike some other phyllodistomes (e.g., *P. singulare*), there are none visible in the adult. The excretory bladder and its contents are the most conspicuous structures of the aging metacercaria. Peculiar "rosette-shaped" refractive granules appear in the cavity of the bladder during the first 24 hours. These granules and the diameter of the bladder both slowly increase in size with age. At all times, however, the granules are clustered in what appears to be a cellular membrane. This membrane may be the outer wall of a "cell" which concentrates the metacercarial metabolic wastes into insoluble concretions. The excretory granules are composed of an insoluble carbonate which gives a pink color reaction with mercuric chloride solution, and which dissolves with the evolution of a gas when treated with an acid.

Little actual progressive development of organ systems takes place during the metacercarial stage. The genital complex, the digestive system and the nervous system all remain at the cercarial level of development.

A 24 hour metacercaria, the youngest infective larva, was 0.25 mm. long by 0.11 mm. wide; the oral sucker was 62 μ in diameter and the acetabulum 64 μ in diameter; the club-shaped excretory bladder was 82 μ long by 34 μ at the widest part. A 61 day old metacercaria was 0.33 mm. long by 0.12 mm. wide; the oral sucker was 70 μ and the acetabulum 79 μ in diameter; the excretory bladder was ovoid, 124 μ long by 92 μ wide.

The length of time spent by trematode larvae in second intermediate hosts, and the degree of differentiation attained in them, often give a clue to the fundamental importance of the host. Certain cercariae (e.g., microphallids) emerge from the molluscan host in a state of relative immaturity and must remain long enough in the succeeding intermediate host to complete their development. Other cercariae (e.g., strigeids) may be apparently completely formed, but in the intermediate host almost complete dedifferentiation occurs, followed by reorganization into new and different larval forms. Still other cercariae (e.g., amphistomes and psilostomes) normally encyst in the open, or within or outside the host, but undergo few, if any, metacercarial changes.

In the gorgoderids we find what may be a partial recapitulation of such an apparent larval phylogeny. *Cercaria duplicata* of Reuss, immediately upon leaving its host, becomes a metacercaria which sinks to the bottom and lies dormant until taken in by the next host. According to Sinitsin (1901), the cercariae of *Phyllodistomum folium* encyst in the parent sporocyst which then emerges from the bivalve and is eaten by the next host. These gorgoderids have an apparent two-host cycle.

Typical three-host cycles also occur in this group of bladder worms. The cercariae of *Gorgodera amplicava*, according to Krull (1935), and the cercariae of *Gorgoderina attenuata*, according to Rankin (1939), are both passively ingested with food by snails and amphibian larvae in which they encyst as metacercariae. The cercariae of *Phyllodistomum solidum*, according to Goodchild (1939a, 1940), and the cercariae of *Phyllodistomum sp.*, according to Crawford (1939, 1940), attract and are eaten by aquatic insect larvae in which they also encyst as metacercariae. Sinitsin (1905), Lutz (1926) and Krull (1935) have also reported finding gorgoderid metacercariae in aquatic insect larvae. Progenetic gorgoderid metacercariae have been found by Wu (1938) in fresh-water shrimps. The bladder flukes reported by Joyeux and Baer (1934), in the abdominal hypaxial muscle of *Rana esculenta*, may also be precocious metacercariae. The intermediate hosts in these three-host cycles, while necessary, are hardly more than vectors which, by serving as food for the definitive host, enable easier completion of the cycle.

6. *Adult.*

The time necessary for this bladder fluke to reach sexual maturity in the final host is correlated with the length of time spent in the second intermediate host; the size increase of a 61-day old metacercaria is comparable to a 15-day old young adult worm developed from a four-day old metacercaria. The temperature at which the urodele is kept may also influence the rate of development of the worm, but as yet, no controlled experimental work has been done on this aspect. These lungless plethodonids require a cool environment for satisfactory maintenance in the laboratory which may partially explain the long period required for sexual maturity of the worm (95 to 130 days).

Practically all development of the fluke takes place postacetabularly. This region increases both in length and width at a greater relative rate than the preacetabular body region.

The accompanying table of measurements (Table 1) shows clearly the increase in size of the body organs while the series of camera lucida drawings (Figures 9 to 14 inclusive) shows relative sizes and growth rates, so that a discussion of progressive development is unnecessary here.

The sexually mature distome (Figures 15, 16 and 17) can be characterized as follows: Length 1.24 to 2.67 mm., width 0.76 to 1.27 mm. The body in fixed specimens is usually not sharply divided into neck and discoidal regions. The oral sucker is large, 0.26 to 0.47 mm. long by 0.27 to 0.48 mm. wide, terminal, and cupshaped; the mouth opening is subterminal. There is no pharynx. The short esophagus, 10 to 90 μ long by 24 to 37 μ wide, bifurcates into narrow crura which immediately expand into the large digestive caeca, 105 μ to 129 μ wide, the anterior lateral edges of which often extend forward to either side of the oral sucker. Posteriorly the caeca reach approximately two-thirds of the distance from the posterior testis to the end of the body. The acetabulum, 390 to 580 μ long by 435 to 560 μ wide, is located between the anterior and middle body-thirds.

The ovary is large, 40 to 280 μ long by 160 to 280 μ wide, ovoid, usually dextral rarely sinistral, located about midway between the acetabulum and the posterior testis, and overlapping the respective gut caecum. The vitellaria consist of a pair of smooth glands lying between the acetabulum and the ovary; the right vitellarium is 78 to 199 μ long by 106 to 163 μ wide; the left vitellarium is 65 to 177 μ long by 110 to 140 μ wide. The vitellaria are connected by a large common vitelline duct, the whole yolk-gland complex appearing dumbbell-shaped. A Mehlis' gland lies at the middle of the common vitelline duct. The oviduct leaves the dorsal side of the ovary and runs antero-mediad; just before it penetrates the shell-gland (Figure 18) it enlarges to form a fertilization space filled with sperm cells; at this same point, Laurer's canal is given off dorsally; it bends anteriorly and opens to the surface dorsally to the shell-gland. The uterus loops over the vitelline duct and bends to the right, at the lateral edge of the acetabulum it turns posteriad and runs to the level of the ovary where it turns mediad between the ovary and posterior testis. The outlines of the uterus are indistinct beyond this point, and the rest of the postacetabular body is filled with developing miracidia. The uterus is visible again, running dorsad and anteriad to the acetabulum, where it expands slightly to form the muscular metraterm which opens into the genital sinus. The ovoid eggs are small, 30 to 31 μ long by 22 to 24 μ wide, and increase in size with the development of the miracidia; the shells are thin and fragile.

The smoothly contoured testes are smaller than the ovary. The anterior testis, 124 to 168 μ long by 186 to 268 μ wide, lies usually in the ovarian field; often, however, its anterior edge may extend to or beyond a level with the anterior edge of the vitellarium. The posterior testis, 88 to 118 μ long by 270

TABLE I

Comparative measurements of Phyllodistomum solidum at various ages and from different sources
All measurements in microns

	One day	Five days	21 days	66 days	95 days	123 days	Mature adult no. 1	Mature adult no. 2	*P. solidum* ex Rankin (1937)	*P. solidum* co-type of Rankin (1937)
Length	209	170	405	513	1102	1445	1460	1240	1820–2670	2120
Width	78	75	126	260	520	685	1000	1040	760–1270	1180
Oral sucker										
Length	47	54	124	200	226	305	286	260	380–470	424
Width	48	47	130	179	243	305	286	273	380–480	453
Acetabulum										
Length	56	55	155	186	325	378	430	390	440–580	459
Width	62	59	163	205	352	390	445	435	510–560	517
Ratio oral to acet. sucker 1:	1.25	1.12	1.25	1.03	1.44	1.26	1.54	1.55	—	1.12
Esophagus										
Length	10	10	54	62	50	47	93	93	10–50	50
Width	4	4	17	18	18	22	25	24	—	37
Gut caeca										
Anterior	4	4	15	24	140	108	129	116	—	109
Posterior	4	4	15	24	109		108	105	—	
Ovary										
Length	10	9	12	39	140	160	160	112	40–280	212
Width	13	10	17	62	148	170	222	225	160–280	212
Vitellaria										
Right									Half the size of the ovary	
Length	5	5	15	23	105	86	140	78		199
Width	8	7	18	40	132	99	121	163		106
Left										
Length	5	5	13	25	108	78	132	65		177
Width	8	8	17	39	124	93	113	140		110
Testes										
Anterior										
Length	7	7	8	30	61	110	124	127		168
Width	15	8	17	66	132	124	186	186		268
Posterior										
Length	7	7	8	47	85	128	93	88		118
Width	15	10	19	59	112	102	270	325		300
Seminal vesicle										
Length					116	80	93	108		
Width					57	62	47	50		
Eggs					28–34 ×22–24	29 ×22	31 ×22	31 ×22	30.4 ×22.4	30.7 ×23.4

to 325 μ wide, located on the ovarian side, is more attenuated transversely than the anterior testis. The vas deferens is located to the left of the metraterm; it expands near the anterior edge of the acetabulum into the conspicuous seminal

vesicle, 93 to 108 μ long by 47 to 50 μ wide. The vesicle also leads into the genital sinus which opens to the outside through the median genital pore.

Discussion

Gorgoderid bladder flukes are usually found in the urinary bladders of their hosts. In *Phyllodistomum solidum*, the young worms after excystment, emerge from the anal opening and crawl about in the cloacal cavity. Eventually they reach the urinary bladder where they reside permanently. The method of orientation of the worm to the proper cloacal opening is unknown; in *Desmognathus* it may be the action of host cloacal cilia. The manner of infestation of fish bladders by gorgoderid trematodes is still experimentally undetermined. Sinitsin (1901), in feeding experiments with metacercariae of his *P. folium*, was able to recover excysted specimens in the intestine of *Carassius vulgaris* and *Abramis brama* two hours after feeding; 24 hours after feeding, he found fewer worms in the urinary ducts. He suggested that young worms migrated from the anal to the urogenital opening; this assumption remains as a distinct probability, and the hazards of an external migration would explain the decrease in numbers of flukes found in the urinary ducts.

The mesonephroi and mesonephric ducts of larger fishes and amphibians have also been reported as additional sites for bladder flukes. Sinitsin (1901, 1905), Nybelin (1926), Lutz (1926), Odlaug (1937) and Rankin (1939) have recovered gorgoderid trematodes from these structures in fishes, toads and frogs. This fact is not surprising because the worms probably feed on the urinary epithelium. In *P. solidum*, cells of an epithelial nature have been seen in sections of gut caeca. The cercarial lytic penetration glands would undoubtedly aid in loosening the epithelial cells which may explain the retention of these glands in some gorgoderid adults. In fish, the "urinary bladder" is merely the dilation or fusion of the posterior parts of the Wolffian ducts; the epithelium is continuous and similar in both ducts and dilations.

Little can be said concerning host-specificity in the group. *Phyllodistomum folium* has been reported from *Carassius carassius*, *Barbus barbus*, *Gobio gobio*, *Scardinius erythrophthalmus*, *Leuciscus rutilus*, *L. cephalus*, *L. idus*, *Aspius rapax*, *Abramis brama* and *A. björkna*, all of which are European Cyprinidae. *P. megalorchis* has been found in *Lota lota*, *Thymallus thymallus* and *Salmo trutta*. *P. simile* has been taken from *Cottus gobio*, *C. poecilopus* and *Thymallus thymallus*. The remaining phyllodistomes are reported from only one or two host species. *P. solidum*, as previously mentioned, was recovered in only one of a group of ten vertebrate species used in feeding experiments. Rankin (1937) reviewed various expressions concerning host-specificity among helminths and stated that the North Carolina gorgoderids have an amphibian host-specificity.

The chaotic taxonomic status of the sexually mature phyllodistomes is due to synonymy and homonymy. Mature worms have few distinctive qualitative differences. Morphological structures which have been used taxonomically are: size and shape; sucker ratio; position of ovary, testes and vitellaria; size of eggs and contours of body organs. These anatomical features do not all remain constant during the maturation of members of the same species. *P. solidum*, as indicated by drawings (Figures 9 to 15 inclusive), passes from a "gorgoderina" shape to a phyllodistome one; the oral-ventral sucker ratio varies greatly in fixed specimens due to pressure differences during fixation (Table I). The eggs likewise vary in length, from 24 to 31 μ and in width, from 14 to 24 μ, depending upon their age.

The synonymies proposed for members of this group should lead one to exercise extreme caution in future gorgoderid taxonomy. Exact taxonomic identification, without knowing the age of the worm, the physical conditions of its host, and the normal size range of the parasite, is extremely difficult. Specimens of *P. solidum* from New York State are usually smaller in all respects than those taken by Rankin in North Carolina. This fact is explained by the longer active period of the southern host, correlated with a probable one year cycle of the mature worm. From observations made in the field, it appears that salamanders are infected with metacercariae in late summer and autumn; worms develop to maturity during the remaining portions of those seasons, and during the winter and early spring. Miracidia are shed in the early spring, infecting clams which give off cercariae not before the first of July. This seasonal cycle would be extended in the milder North Carolina weather, allowing time for the worms to grow larger.

The caenogenetic organs of the cercariae offer more promise for positive specific identification. Finally, life-cycle studies, using as many different hosts as possible, may settle questionable synonymy.

Typical life-cycles were formerly believed to be family characteristics, similar in their deep-seated significance to the structures and arrangements of adult organs. In the Gorgoderinae, the life-histories so far reported, have varied greatly both in larval structures and typical sequences. The non-stylet, stumpy-tailed cercaria described by Sinitsin (1901), encysts in the sporocyst which then emerges and being remarkably buoyant because of fat-filled columnar wall cells, rises with sluggish wriggling movements to the surface, where it is eaten by fish. Dobrovolny (1939) reported the same type of life-cycle for *Plagioporus sinitsini*, a small allocread trematode from the gall-bladders of fresh-water fish. He also commented on the loss or suppression of the free-swimming cercarial stage which has been observed in other trematodes.

The non-stylet rhopalocercous gorgoderid *Cercaria duplicata* of v. Baer (1827) and Reuss (1903) emerges from its bivalve host and almost immediately the anterior portion of the large cercarial tail distends anteriorly to enclose the distome in a tail chamber. In the more typical gorgoderid cercariae, the same enclosing mechanism is effected precociously in the sporocyst. The stage represented by Reuss in Figure 4, if complete, does not represent an encysted gorgoderid metacercaria; he shows the distome body free in the tail chamber without any indication of a true enclosing cyst membrane. This fact may explain Reuss' inability to get further development of the worm in feeding experiments with any of 16 different species of fish.

Nybelin (1926) reported the encysted metacercariae of *P. megalorchis* from the fish, *Phoxinus sp.* Identification of the trematode was circumstantially based on the presence of only this one known species of sexually mature phyllodistome in the same waters. The stylet cercaria was not found.

Arthropods have also been reported as intermediate hosts of gorgoderid trematodes by Sinitsin (1905), Lutz (1926), Krull (1935), Wu (1938), Crawford (1939, 1940) and Goodchild (1939a, 1940).

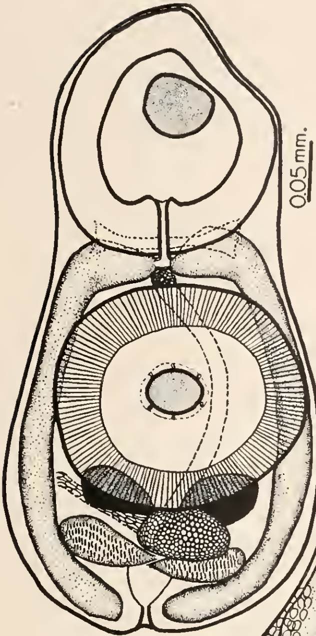

FIG. 12

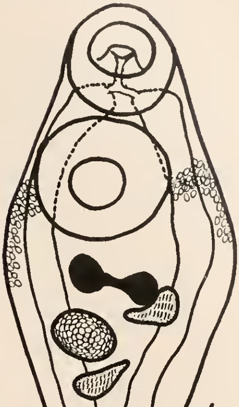

FIG. 14

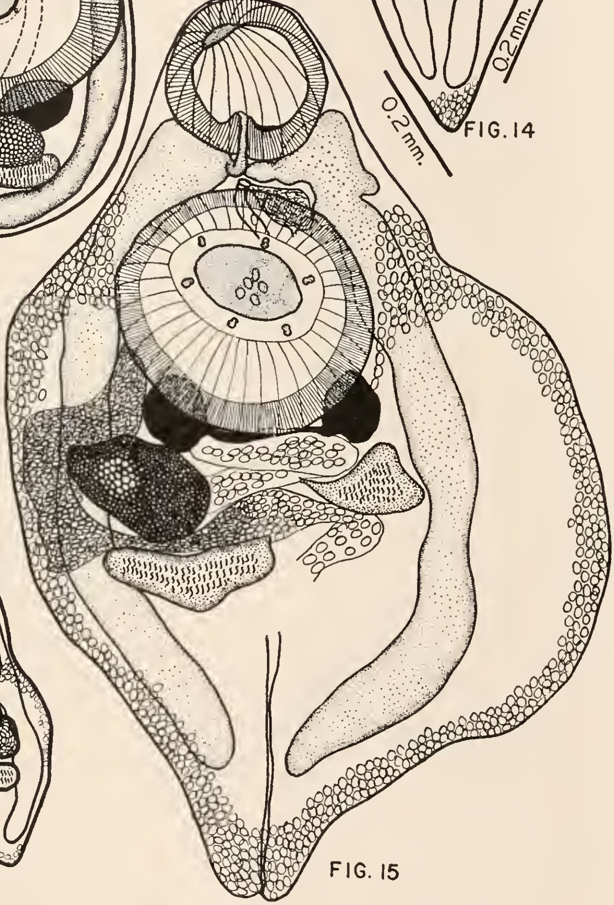

FIG. 15

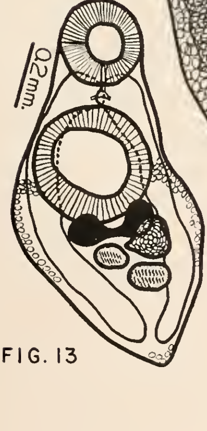

FIG. 13

PLATE 3

FIGURE 12. Young adult (66 days).
FIGURE 13. Young adult (95 days).
FIGURE 14. Young adult (123 days).
FIGURE 15. Mature *Phyllodistomum solidum.*

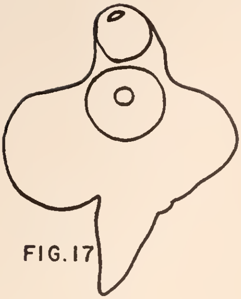

FIG. 17

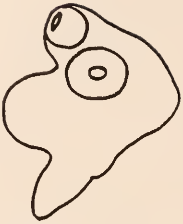

FIG. 17

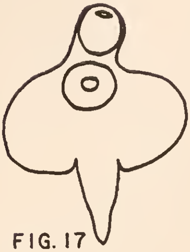

FIG. 17

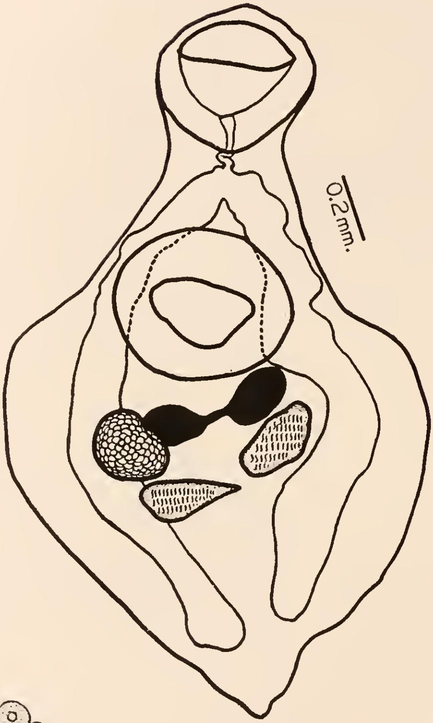

FIG. 16

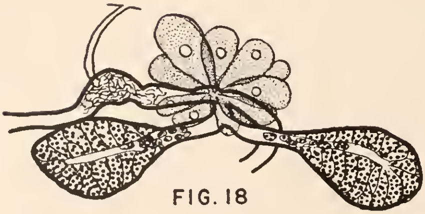

FIG. 18

PLATE 4

FIGURE 16. Co-type, *P. solidum* of Rankin.
FIGURE 17. Shapes of living *P. solidum*.
FIGURE 18. Genital complex of adult.

Sinitsin (1905) described inclusive life-cycles for *Gorgodera pagenstecheri* and *Gorgoderina vitelliloba*, while for *Gorgodera cygnoides* and *Gorgodera varsoviensis*, he conducted incompletely controlled life-history studies. In all four of these species odonatan naiads and beetle larvae served as second intermediate hosts. Lutz (1926) reported from South America that macrocercous gorgoderid cercariae when eaten by odonatan larvae penetrated the esophagus in the wall of which they encysted. Wu (1938) reported the progenetic metacercariae of *P. lesteri* from Chinese fresh-water shrimps, *Palaemon asperulus* and *P. nipponensis;* he was unable to determine the host of the adult worm. Crawford (1939, 1940) found that odonatan larvae, caddice-fly larvae and diving-beetle larvae served as the second intermediate hosts of *P. americanum*. Goodchild (1939a, 1940, and in this paper) has reported odonatan nymphs as intermediate hosts of *P. solidum*. Krull (1933, 1935) determined that the cercariae of *Gorgodera amplicava* was ingested by the snail, *Helisoma antrosa* in which it became an infective metacercariae. Krull (1935) reported that "frog bladder fluke cercariae" were ingested by the nymphs of damsel-flies, *Lestes sp.*, and formed metacercariae in the body cavity.

Rankin (1939) determined that tadpoles of *Rana pipiens*, *R. clamitans* and the snail, *Pseudosuccinea columella* serve as the intermediate hosts of *Gorgoderina attenuata*.

Gorgoderids with two-host cycles and those with three-host cycles would appear to be distantly related. It is possible to assume, however, that there is present in this group, the fortunate retention of intermediate forms in a rather complete evolutionary series. The phylogenetic modifications and specializations of the life-cycle have not altered the fundamental morphologies of the adults or the preference for their customary parasitic sites.

Transitions Between Genera in the Gorgoderinae

1. *Gorgodera* and *Gorgoderina*.

These genera have been accepted as valid since Looss (1902) separated them on the basis of the number of testes, of which the former had nine, the latter two. Rankin (1939) showed that metacercariae of *Gorgoderina attenuata* have nine testes (six on one side, three on the other) which gradually fuse to form two in the maturing adult. In the same paper, he concluded that although these two genera of worms are definitely separable, nevertheless, "the genera *Gorgodera* and *Gorgoderina* are apparently very closely related, not only morphologically, but also with respect to their life cycles and modes of development." Krull (1935) reported that in one of the largest metacercariae of *Gorgodera amplicava* a posteriorly extended string of cells showed enlargements which were the primordia of testes. However, in young adults the, "dense testicular mass shows little evidence of being subdivided into testes, and there is little separation into right and left parts until maturity." This testicular condition again reveals the closeness of these two genera. Odlaug (1937), reporting on young kidney stages of *Gorgodera amplicava*, showed early separation into testicular masses. Although Odlaug's interpretation of the number of these units was probably erroneous, in that the ovary and vitellaria were interpreted as testes, nevertheless, the early testicular segregation that he found is significant.

2. *Gorgoderina* and *Phyllodistomum.*

For opinions regarding the identity of these two genera see: Osborn (1903), Sinitsin (1905), Cort (1912), Nybelin (1926), Ozaki (1926), Pande (1937) and Byrd, Venard and Reiber (1940). Crawford (1939, 1940) described a bladder fluke from *Bufo boreas boreas* and *Amblystoma tigrinum* which is intermediate between the two genera. This worm has a phyllodistome-like postacetabularly flared body, but also possesses prominent uterine coils between the vitelline complex and the acetabulum (*P. acceptum* and *P. marinum* have similar morphological traits), a condition regularly found in the genus *Gorgoderina.* In its early adult development this worm also passes through a typical Gorgoderina shape.

Gorgoderina schistorchis Steelman, 1938 and *Gorgoderina tenua* Rankin, 1937 possess phyllodistome morphology and they should be included in the genus *Phyllodistomum.*

Because of limited knowledge of life-histories in these genera it is unwise, at present, to suppress dogmatically one of them as a synonym of the other.

3. *Phyllodistomum* and *Catoptroides.*

For a discussion of the history of these two genera consult: Lewis (1935) and Byrd, Venard and Reiber (1940).

Nybelin (1926), Lewis (1935), Lynch (1936), Bhalerao (1937), Wu (1938) and Steelman (1938) have advocated, upon morphological bases, the suppression of *Catoptroides* as a synonym of *Phyllodistomum.* On the other hand, Loewen (1929, 1935), and Arnold (1934) upheld generic separation and described new species in the genus *Catoptroides.* Recently, Byrd, Venard and Reiber (1940), upon the basis of the excretory system, have re-established the frequently suppressed *Catoptroides.* The reasons for this action were: (1) differences in the position of the main collecting duct bifurcation (anterior to the acetabulum in the *Gorgodera-Catoptroides* group, and posterior to the acetabulum in the *Phyllodistomum-Gorgoderina* group), (2) the manner in which the collecting ducts unite with the bladder, and (3) the way in which the accessory tubules arise from the main collecting tubules.

Byrd *et al* (1940) found for *Gorgodera amplicava, Catoptroides lacustri* and *Phyllodistomum lohrenzi,* and Olsen (1937) found for *Gorgoderina tanneri* a constant flame-cell pattern of 2 × 8 × 4 = 64 flame-cells. In their papers they did not discuss the data which has been presented concerning the bifurcation of the main collecting ducts in other species of Gorgoderinae.

The majority of gorgoderid species possess main collecting ducts which run anterior to the acetabulum before bifurcation. Sinitsin (1905) reported such a condition for the cercariae of *Gorgodera varsoviensis, Gorgodera cygnoides, Gorgodera pagenstecheri* [Wesenberg-Lund (1934) figured this last cercaria and showed bifurcation of the main collecting ducts at the level of the anterior-third of the acetabulum], and *Gorgoderina vitelliloba* [Wesenberg-Lund (1934) figured postacetabular bifurcation in this species of cercaria, but wrote (p. 96), "two excretory canals issue from the anterior part; near the middle of the ventral sucker they seem to divide into an anterior and posterior branch."] The account given by Vickers (1940) concerning the cercaria of *Gorgoderina vitelliloba* supports the observations of Sinitsin.

The following authors have also reported an anterior bifurcation of the main collecting ducts: Reuss (1903) in *Cercaria duplicata;* Sturges (1897) in *Phyllodistomum patellare;* Krull (1935) in *Gorgodera amplicava;* Miller (1935) in *Cercaria sphaerocerca;* Steen (1938) in *Phyllodistomum brevicecum.* Wu (1938) stated that in *Phyllodistomum lesteri,* "the two tubules pass through the respective vitellaria and go around the ventral sucker." Steelman (1939) remarked for *Cercaria coelocerca,* "a pair of much-convoluted lateral collecting tubules extends anteriad from front end of bladder to near level of brain before branching." Goodchild (1939a, 1939b) found branching of the main collecting ducts anterior to the acetabulum in the cercaria of *Phyllodistomum solidum* and *Cercaria donecerca.* Vickers (1940) in a very detailed morphological investigation of *Cercaria macrocerca* Fil. (= *C. Gorgoderina vitelliloba*) described lateral excretory ducts which proceeded anterior to the acetabulum before dividing. The excretory system as described by Vickers for the cercaria is not complete, but the reported arrangement does not fit any gorgoderid pattern so far determined. Fischthal (1942) in describing *Phyllodistomum semotili, P. notropidus* and *P. nocomis* stated that the right and left primary collecting ducts are seen, "extending anteriorly to intestinal bifurcation, then looping posteriorly a short distance each receiving two secondary collecting ducts."

Postacetabular bifurcation of main collecting ducts has also been reported in a few gorgoderid species. Byrd *et al* (1940) have found this type in *Phyllodistomum lohrenzi.* Rankin (1939) reported posterior bifurcation in *Gorgoderina attenuata;* and Olsen (1937) found it in *Gorgoderina tanneri.* Walker (1937) found in *Gorgoderina vitelliloba* a median excretory vesicle extending forward to the anterior border of the posterior testis. He stated further, "here it bifurcates into two lateral canals which diverge from one another and extend forwards externally to the intestinal caeca until opposite the anterior testis, where each canal divides to form two branches, one lying dorsally to the other. The ventral branch extends forwards to the level of the acetabulum, while the dorsal extends in front of the acetabulum."

A final type of bifurcation was described by Miller (1936) in *Cercaria mitocerca.* Here, the main collecting ducts divide into an anterior and posterior branch at the level of the middle of the acetabulum.

Because there is no uniformity in the literature concerning excretory patterns of the same species (*Gorgoderina vitelliloba*), or members of the same genus, and because definite transition forms (*Cercaria mitocerca*) occur between these two different bifurcation types, it is evident that conclusions drawn now from such data are premature and questionable.

Conclusions and Summary

1. The life-history of *Phyllodistomum solidum* Rankin, 1937, a gorgoderid trematode from the urinary bladder of the urodele, *Desmognathus fuscus fuscus* (Raf.), has been completed experimentally in the laboratory.

2. *Cercaria Phyllodistomum solidum* (= *Cercaria conica* Goodchild, 1939) from *Pisidium abditum* Haldeman by vigorous cercarial activity attracts and is eaten by odonatan naiads (*Ischnura verticalis, Argia sp., Enallagma sp., Libellula sp.*) which serve as metacercarial hosts.

3. Metacercariae encyst in the thoracic haemocoele of the insect within four minutes of ingestion and are infective for the final host after 24 hours.

4. In the salamander, the metacercariae excyst in the small intestine and are recoverable in the intestine, cloaca, "urethra" and urinary bladder within 24 hours after ingestion of the infective metacercariae.

5. In the laboratory these bladder flukes require three months time to attain sexual maturity. The mature worms produce eggs which hatch immediately liberating free-swimming miracidia.

6. The miracidia are drawn by water currents into the gills of the bivalve where they transform into mother sporocysts. Mother sporocysts each produce a single generation of daughter sporocysts which, in turn, give rise to the large-tailed gorgoderid cercariae.

7. From miracidial penetration to cercarial production requires three months time in the laboratory.

8. A key to the gorgoderid cercariae is included.

9. The phylogeny and life-cycles of non-stylet and stylet gorgoderid cercariae are discussed.

10. *Gorgoderina tenua* Rankin, 1937 and *Gorgoderina schistorchis* Steelman, 1938 have been placed in the genus *Phyllodistomum*.

11. Comparisons between the genera: *Gorgodera* and *Gorgoderina*, *Gorgoderina* and *Phyllodistomum*, and *Phyllodistomum* and *Catoptroides* are discussed.

12. Observations on the gorgoderid excretory system as a basis for taxonomy are reviewed.

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THE REPRODUCTIVE CYCLE OF THE VIVIPAROUS TELEOST, NEOTOCA BILINEATA, A MEMBER OF THE FAMILY GOODEIDAE. IV. THE GERMINAL TISSUE

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INTRODUCTION

The present article is the fourth of a series written by the author on the reproductive cycle of the viviparous cyprinodont, *Neotoca bilineata*. The series has been an attempt to present a more complete study than has been available heretofore on the reproductive cycle of a viviparous teleost. Previous studies by other investigators have been confined largely either to the breeding cycle or to histological descriptions of the ovary, stressing particularly the adaptation of the ovary to the retention of the young during development. Little or no attention had been given to the actual changes in the ovarian soma during gestation until the papers on *Jenynsia* (*Fitzroyia*) *lineata* (Hylton Scott, 1928; Siccardi, 1940), *Xiphophorus helleri* (Bailey, 1933), *Neotoca* (*Skiffia*) *bilineata* (Turner, 1933), and *Cymatogaster aggregatus* (Turner, 1938b). Some of the above papers were merely introductory descriptions; others were more complete. Furthermore, with the exception of a study of the poeciliids by Turner in 1937, no reference had been made even to the cyclic variation of the germ cells during gestation. Consequently, the writer undertook this investigation with the purpose of presenting in detail an analysis of the reproductive cycle stressing particularly some of the phenomena generally omitted by previous investigators. Articles by the writer on the reproductive cycle of Neotoca have described in detail (1) the breeding cycle as observed in the laboratory (1939), (2) the marked cyclic changes in the ovarian soma during gestation (1940), and (3) the variations in the germ cell count during gestation (1941). The present article, the fourth of the series, supplements brief preliminary descriptions (Turner, 1933; Mendoza, 1938) by considering in detail (1) the general description and growth of the germ cells and their follicles, (2) fertilization, (3) the fate of the evacuated follicles, (4) the nature and fate of the atretic follicles, and (5) the origin of germ cells in the adult ovary.

MATERIALS

The present description of the germinal tissue is based on a study of over 30 ovaries in different stages of gestation. The greater part of the material was fixed in Bouin's or Zenker's fluids although fixatives such as Flemming's fluid, osmic acid, and corrosive sublimate were used for special techniques. The principal stains used were iron hematoxylin and Delafield's hematoxylin followed either by Eosin Y or Orange G. In addition, the following special stains were used: Mallory's triple connective tissue stain, Van Geisen's stain, and Foot's modification of Bieslchowsky's silver impregnation technique. The customary alcohol-xylol dehydration series was used.

OVARY

The ovary of Neotoca is a single, spindle-shaped organ inserted in the median sagittal line; it is attached dorsally to the pleuroperitoneal membrane and ventrally to the mesogaster. The ovary is continuous caudad into a single short gonoduct, a term preferred by Turner and others since, evidently, it is not homologous to the true vertebrate oviduct. Neotoca is similar to most viviparous teleosts since almost all possess the single median gonad; only in forms such as *Sebastodes rubrovinctus* (Eigenmann, 1892) is the ovary double, and only in few forms such as *Dermogenys pusillus* (Peters, 1865), *Lucifuga subterraneous* and *Stygicola dentata* (Lane, 1903), is the ovary partially fused. So far as is known to the writer, all viviparous teleosts have the single gonoduct even in forms that have the double or partially fused gonads.

GERMINAL TISSUE

Ovigerous folds

The ovary of a teleost may be a solid or hollow organ. In the former case the eggs merely rupture the wall of the gonad, fall into the coelom and then escape to the outside through abdominal pores; in the latter type, the eggs never reach the coelom but make their way out directly from the ovary through the gonoduct. In Neotoca the gonad is hollow and the germinal tissue is said to occur internally; the same condition occurs in all other viviparous teleosts known to the writer.

The oöcytes are not scattered at random but are confined to "ovigerous" or egg-bearing folds that are placed on either side of a median sagittal septum.

PLATE I

FIGURE 1. A cross-section of a typical non-gravid ovary (19.5×). Part of the ovarian wall at the lower left-hand corner has been removed. In the center of the figure, in a nearly vertical position is the median septum of the ovary. On either side of the septum lie the two ovigerous folds attached by a narrow, constricted stalk to the ovarian wall. The attachment is particularly clear on the left side. The arrow points to the lower edge of the ovigerous fold on the left side. Prominent in the photograph are several developing oöcytes.

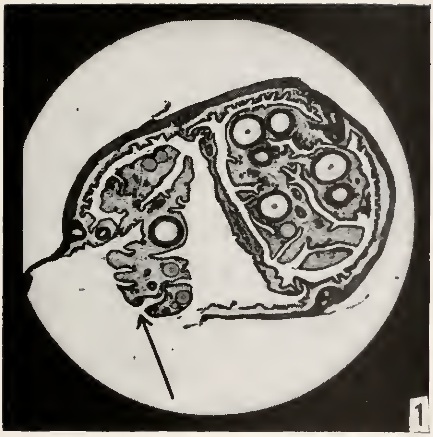
FIGURE 2. A growing oöcyte. The photograph shows clearly the nucleolus, the vesicular nucleus, and the early follicle composed of squamous cells.

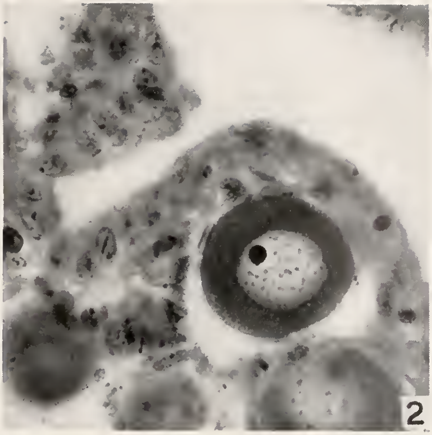
FIGURE 3. Two good examples of developing oöcytes and their follicles. The medium size oöcyte in the upper portion of the figure shows a nucleus that has lost its vesicular character and has become quite granular. The thickened follicle is the result of rapid mitoptic activity that does not cease until the follicle cells become so abundant they form a compact layer of columnar cells as in the egg at the bottom of the figure.

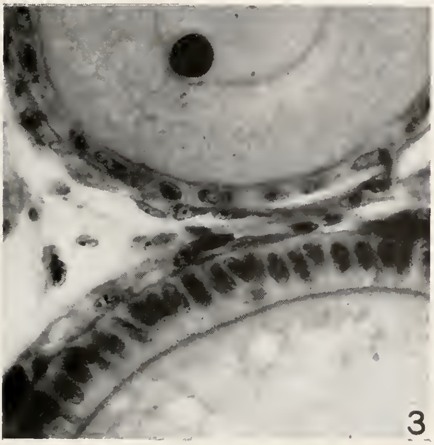
FIGURE 4. A figure that has a two-fold value. First, it shows an oöcyte and its follicle in a stage of development intermediate between that of the two eggs in the preceding figure. The follicle cells now are cuboidal to low columnar and soon will attain the full columnar shape typical of the full grown follicle. Secondly, the figure shows the vacuolated yolk-nucleus (arrow) in a medium size oöcyte. Figures 2 to 4 are intended to show the sequence of changes in the oöcytes and their follicles during growth.

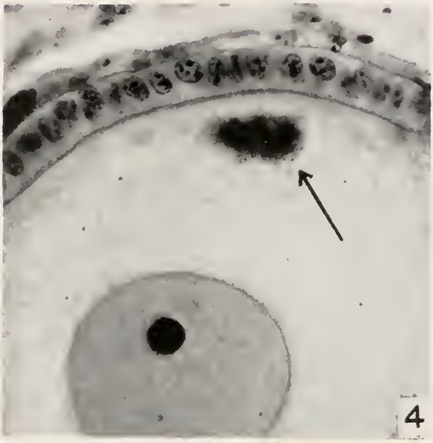
FIGURE 5. A typical delle showing the funnel-like depression of the ovigerous fold epithelium that reaches to the follicle of the egg at the lower left. The intimate contact between the ovigerous fold epithelium and the follicle cells is clearly visible. Within the delle are two spermatozoa (arrows). In the upper left-hand corner is a typical mitotic figure in a growing follicle.

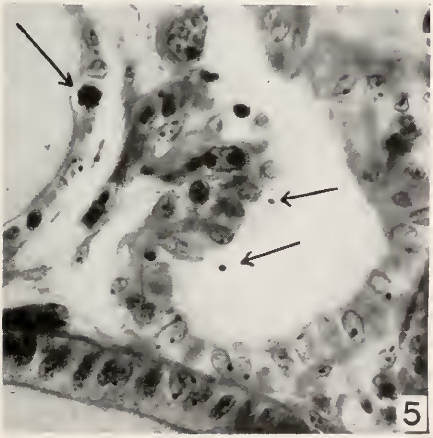
FIGURE 6. A high-power photograph (283×) of a follicle after the expulsion of the egg. The position of the former delle is visible still at the upper left-hand corner, showing the place through which the egg escaped from the follicle and the ovigerous fold. The follicle cells still retain their peripheral position although degenerative changes already have started.











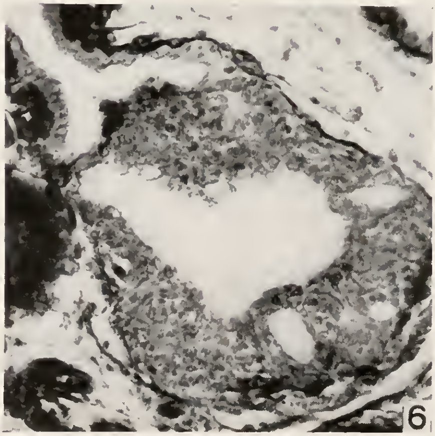


PLATE I[1]

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[1] All figures are contact prints of unretouched photomicrographs. Figure 1 was taken with a Reflex-Korelle camera; all others were taken with a Voigtlander cut-film camera. With exception of Figures 1 and 6, all other photographs were taken with a fluorite, oil-immersion objective and a Hyperplane ocular; the magnification of the oil-immersion photographs is approximately 650×.

Each fold is attached by a narrow strip along the dorso-lateral wall of the gonad; the two folds virtually fill the ovarian lumen (Figure 1). Internal ovigerous folds by no means are characteristic of all viviparous teleosts since *Xiphophorus helleri*, *Anableps anableps*, and *Zoarces viviparus* are devoid of special folds; the ova merely develop in the walls of the ovary. On the other hand, forms such as *Cymatogaster aggregatus*, *Dermogenys pusillus*, *Jenynsia lineata*, *Sebastes marinus*, etc., resemble Neotoca and other members of the Goodeidae by the presence of one type or another of ovigerous folds or septa. In fact, these folds are so diagnostic in the Goodeidae that Hubbs and Turner (1939) used these same folds, in part, as a basis for their recent taxonomic revision of the family.

Histologically, the folds consist of a loose, collagenous connective tissue that in part shows a marked affinity for argyrophilic stains. A vascular network ramifies throughout the folds and forms a very rich capillary plexus under the epithelium of the folds. It has been shown (Mendoza, 1940) that during gestation, this internal epithelium covering the folds changes from a flattened, indifferent condition to a tall, highly secretory epithelium. Parallel with these changes, the folds likewise become distended with fluid and are invaded by numerous free cellular elements that contribute to the general tumescence of the ovary during gestation. Within the ovigerous folds of Neotoca, the germ cells are evenly distributed throughout the length of the gonad except at the extreme anterior and posterior ends. The growing germ cells are not scattered at random but are normally grouped into clusters of cells that range from minute oöcytes 7 to 10 μ in diameter to fully grown eggs approximately 200 μ in diameter. In addition, these clusters of cells normally occur at the surface of the folds, retaining a very intimate connection with the epithelium (Figures 2, 10, 12), a relationship that was stressed also for Anableps by Turner (1938a). At this point of contact between the fully grown ovum and the epithelium, there appears a deep, funnel-like depression of the ovigerous fold epithelium. The cells at the base of the pocket are usually flattened strongly against the follicular cells surrounding the ovum (Figures 5, 9). Normally this depression does not occur at the outer surface of the folds but at the base of one of the many fissures that crease the surface of the folds. This funnel-like depression has been described in other viviparous teleosts and has been identified as a "tubular indentation," a "follicular pore," or a "delle." The two-fold function of this structure will be discussed elsewhere in this paper.

Germ cells and follicles

Germ cells are recognizable when they are but 7 μ in diameter and are undergoing early stages of maturation. Such early cells are grouped into small nests and usually are attached closely to the ovigerous fold epithelium. In these early cells the nucleus is vesicular and occupies fully two-thirds or more of the diameter of the growing oöcyte. The cytoplasm shows an affinity for acidophylic stains and is heavily but evenly granular (Figure 2). The early follicle consists of a few delicate, squamous cells flattened against the surface of the oöcyte (Figure 2); because of the manner of formation of the germ cell nests and the structure of the follicle cells, it is likely that the latter are nothing more than modified fibrocytes of the subepithelial connective tissue.

In the growing oöcyte, furthermore, many changes occur. The nucleus is reduced to one-third of the diameter of the cell and the vesicular character is lost; it becomes granular and even oxyphylic in nature (Figures 2 to 4). During growth, the chromatin loses all affinity for stains although it later reappears in the form of lampbrush chromosomes. Whereas in the early cells the nucleoli may be numerous, less than a micron in diameter and strongly basic in reaction, later they are but few in number, large, vacuolated, and even oxyphylic in their reaction to stains. In general, however, their number, size, and appearance are highly variable in the different cells and at different stages of development. In the growing cells the cytoplasm also undergoes marked changes. The most conspicuous characteristic of the oöcytes is the complete absence of large masses of yolk and the presence of numerous small droplets of oil scattered throughout the granular cytoplasm. The yolk, such as it is, consists largely of a granular, flocculent mass evenly distributed throughout the cytoplasm and very difficult to distinguish except with the use of differential stains and particularly in stages immediately following fertilization. There is not the least similarity to forms such as Zoarces in which the yolk appears as large spheres, nor to Jenynsia and Xiphophorus in which the yolk appears as a large solid mass. Furthermore, another goodeid, *Lermichthys multiradiatus*, shows a heavy yolk mass in the developing embryos, a mass that, though small in size, is so heavy and compact, it cannot in any way be compared to the "yolk" of Neotoca. It is regrettable that lack of material prevented further testing or differentiating of fatty and proteinaceous yolk in Neotoca although the use of Flemming's fixative on the ova of another goodeid, *Girardinichthys innominatus*, showed beyond doubt that there is a large number of droplets or spheres of fatty yolk concentrated around the nucleus of the ovum. Another conspicuous feature of the fully-developed ovum is a large, vacuolated structure that is very similar to or forms a yolk-nucleus and pallial layer complex (Figure 4). The origin, development, and fate of this yolk-nucleus complex is so interesting in these viviparous forms that it will be discussed at length in a separate paper. It is interesting that, among viviparous teleosts, Cymatogaster and Jenynsia have yolk nuclei equally as prominent as that of Neotoca. The egg membranes of the enlarged oöcytes are not numerous; there is primarily one heavy vitelline membrane densely perforated in part, if not in its entirety, by minute pores. Thus it is similar, though not identical, to the thick, perforated, zona radiata described for Zoarces by Stuhlmann and for Cymatogaster by Hubbard (1894). Internal to this heavy egg membrane is a more delicate plasma membrane discernible only after fertilization when the heavy outer membrane is separated somewhat from the egg. During growth of the oöcyte, the mitotic activity of the follicle cells not only keeps pace with but actually surpasses the growth of the egg since the follicle changes from a delicate layer of flattened cells to a single, densely-packed row of columnar cells (Figures 2 to 5), a follicle that more closely resembles that of Xiphophorus and the early follicle of Anableps. In comparison, follicles of a compound nature are found in forms such as Jenynsia, Stygicola, Lucifuga, and Cymatogaster. The follicle in Neotoca is in turn invested by a thin layer of condensed connective tissue fibers and fibrocytes comparable to the "theca" described by Bailey for Xiphophorus. The connective tissue fibers of this "theca" are intimately associated with those of the subepithelial network of the ovigerous folds and, like reticular fibers, similarly show a marked affinity for argyrophylic stains. Interspersed throughout the interstices of this network there occurs a vascular network more or less prominent in the different follicles and presumably of great importance in the physiological activity of the follicle of the developing oöcyte.

Fate of the Germ Cells

After the ova have attained full growth they suffer one of two fates, either they are fertilized and commence development or they undergo atresia. Both possibilities are considered.

Fertilization

Among viviparous teleosts, fertilization may occur while the egg still is retained within the follicle or after its extrusion into the ovarian lumen. In forms such as Anableps and Xiphophorus, fertilization occurs within the follicle and the embryo is not released until a short time preceding birth whereas in Jenynsia, fertilization is similar but the embryo is discharged early in development. On the other hand, in Cymatogaster, *Sebastes marinus* (Williamson, 1910), and Neotoca, fertilization and the expulsion of the egg must occur in such rapid succession that it is difficult to separate the two phenomena. Finally, in Zoarces, eggs are discharged first into the ovarian lumen and fertilized later. Accompanying these varying conditions of fertilization, structural and functional problems are presented by the different types, problems that are largely beyond the scope of this paper.

Preceding fertilization in Neotoca, the different stages of meiosis can be identified readily. Following synizesis which appears in oöcytes 7 to 10 μ in diameter, the chromatin temporarily loses all affinity for stains and later reappears in the form of lampbrush chromosomes. Immediately preceding actual fertilization the chromatin condenses markedly, resembling stages of diakinesis, while the entire germinal vesicle migrates simultaneously toward the periphery of the egg. This migration occurs normally in the direction of the delle, in preparation for the ensuing meiotic divisions and fertilization. Evidently, the meiotic divisions, fertilization, and the expulsion of the egg into the ovarian cavity must occur simultaneously or in very rapid succession, for none of the three phenomena actually have been seen although all stages immediately preceding and following their occurrence have been identified. Unfertilized eggs always have been identified within the follicles; on the other hand, fertilized eggs normally have not been seen within the follicles but always free in the ovarian lumen. The criterion used to distinguish fertilized eggs has been the very radical displacement of cytoplasmic components within the egg and the ensuing segmentation. In these eggs, the cytoplasm is concentrated as a thin peripheral layer, presumably along the animal pole; small oil droplets occur evenly distributed throughout its extent. Underneath the thin blastodisc, the central portion of the fertilized egg is filled by a flocculent, albumen-like substance which apparently replaces the heavy yolk of other viviparous eggs. Occasional spheres of actual yolk can be found, but they are minute and extremely scarce. Although the meiotic divisions have not been seen, the polar bodies have been identified on the edge of the heavy vitelline membrane. A unique phenomenon is that in the vicinity of the polar bodies the vitelline membrane not only shows a strong affinity for basic stains but also thickens conspicuously at time of fertilization to resemble the typical zona radiata of teleost eggs. Since this thickening of the membrane occurs only in evacuated eggs and only near the polar bodies, it is assumed that it arises in response to the inciting action of the spermatozoa. Due largely to the swelling of the vitelline membrane of these eggs, it can be distinguished from the egg with ease.

It is at the time of fertilization that the delle formed by the ovigerous fold epithelium plays an important role. Through the delle, the spermatozoa actually can approach the egg very closely (Figures 5, 9). If the sperm do, in fact, enter the egg while the latter still is within the follicle, the sperm then must penetrate only through the single row of flattened cells at the base of the delle and the row of columnar follicle cells. However, the writer never has identified an actual pore within the follicular epithelium to permit the entry of the spermatozoa as described for Xiphophorus by Bailey. Evidence is available from other viviparous teleosts to show that fertilization within a follicle can and does occur. In Xiphophorus, Anableps, Cymatogaster and Jenynsia, eggs are fertilized while still enclosed within the follicle and later are discharged into the ovarian lumen at different stages of development. The second and final function of the delle is that it offers an attenuated and weakened place in the ovigerous fold epithelium through which the egg escapes into the ovarian cavity. A similar role for the delle has been reported for other viviparous teleosts in which it appears.

Upon evacuation of the egg, the follicular cells cease all mitotic activity and suffer one of two or three fates. Sometimes the force of the expulsion of the egg is so great that the follicle cells may be everted part way through the delle. Normally, however, the follicle cells either merely collapse to form a cellular mass of smaller diameter or the follicle cells may retain their peripheral position around the reduced space previously occupied by the egg (Figure 6). Despite these differences in evacuated follicles, the cells eventually lose their regularity and become a disorganized mass of cells. The evacuated follicle does not assume the "windswept appearance" of the Xiphophorus follicle (Bailey) nor does it become hypertrophied as in *Sebastes marinus* and *Anableps anableps*. It is impossible to compare the spent follicles of Neotoca with the mammalian corpora lutea since in Neotoca there is no evidence of a pronounced physiological activity; actually, the follicle cells are small and shrunken. Pycnotic figures of degeneration make their appearance soon after the extrusion of the egg; vacuolization and fatty degeneration have been found among these follicles (Figure 6). By the time embryos are 1.5 mm. in length it is difficult to distinguish between spent follicles and ordinary atretic follicles. It is to be expected, perhaps, that in Neotoca the follicle should be passive and soon degenerate for, since the embryo develops completely within the ovarian lumen, the follicle is relieved of all responsibility of serving the developing embryo in a nutritive or respiratory capacity. Thus the Neotoca follicle is not taxed with a physiological burden similar to that of Anableps nor poeciliids like Xiphophorus in which the embryo develops completely within the follicular sac.

Atresia of the eggs and follicles

Egg degeneration is found in all ovaries. It is evident that eggs degenerate rapidly if not fertilized soon after they have attained full growth. As many as 25 eggs in various stages of atresia have been found in a single non-gravid ovary. In typical cases of degeneration which occur during all stages of gestation, both the follicle and the egg are involved. Normally, the egg and especially the cytoplasm is the first to disintegrate; the alveolar structure is lost and frequently the cytoplasmic residue coagulates upon fixation. Coexistent with these changes, the follicle cells lose their regularity, mitosis ceases, the vitelline membrane is thrown into folds, and the follicle cells are displaced toward the atretic egg. Normally, however, the follicle cells retain their peripheral position for some time while the cytoplasm of the egg breaks up. It is likely that the debris of the egg is removed in part by absorption and in part by actual phagocytosis. With the removal of most of the cytoplasmic residue, the follicle collapses completely and forms a nearly solid group of cells surrounding a mass of debris (Figures 7, 8). During this process, slight lymphocyte infiltration usually occurs and may be instrumental in aiding the process of disintegration. In the follicle cells, the nuclei are the first to undergo pycnotic degeneration; the breakdown occurs gradually, cell by cell. The degeneration picture most definitely is not one of complete breakdown of all or most cells at once. In a few isolated cases an abnormally heavy lymphocyte infiltration may occur and in others the follicle may precede the egg in degeneration. These, however, are infrequent in their occurrence. Eventually, regardless of the method of atresia, the end result is the removal of the degenerating mass of cells from the stroma of the ovary.

Origin of the Germ Cells

Although no study has been made of the origin of germ cells in the embryonic gonad, the writer feels that there is available interesting evidence on the origin of the cells in the adult gonad. The observation has been made repeatedly that

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Plate II

Figure 7. An atretic follicle showing the disorganized state of the follicle cells, the cellular debris, some fatty degeneration, and several large vacuoles that have appeared between the cells.

Figure 8. A degenerating follicle that has been reduced to a small cellular mass. In the center there occurs some cellular debris and one atretic cell (arrow).

Figure 9. This and the remaining figures on the plate have the single purpose of showing the frequent occurrence of growing oöcytes in the ovigerous fold epithelium and the possible origin of some germ cell nests from the epithelium. The arrows indicate clearly an oöcyte in the epithelium that lines the delle and three spermatozoa within the delle proper. This and Figure 5 show the relation of the ovigerous fold epithelium of the delle to the egg and its follicle.

Figure 10. The arrows indicate first, a developing oöcyte in the ovigerous fold epithelium and second, a new germ cell nest apparently derived from the epithelium.

Figure 11. A single, distinct oöcyte in the epithelium of the ovigerous folds. The subepithelial connective tissue, showing black in the photograph, indicates clearly that the germ cell is in the epithelium and not merely lying against it.

Figure 12. The opening that shows in the center of the photograph is a cross-section of a delle. The arrows indicate two oöcytes within the epithelium of the delle and other germ cells that apparently have broken through the subepithelial connective tissue fibers to form a small clump or nest of growing oöcytes. The photograph shows clearly how the basement membrane has been ruptured completely at this point, indicating a complete continuity between the growing oöcytes and the ovigerous fold epithelium.

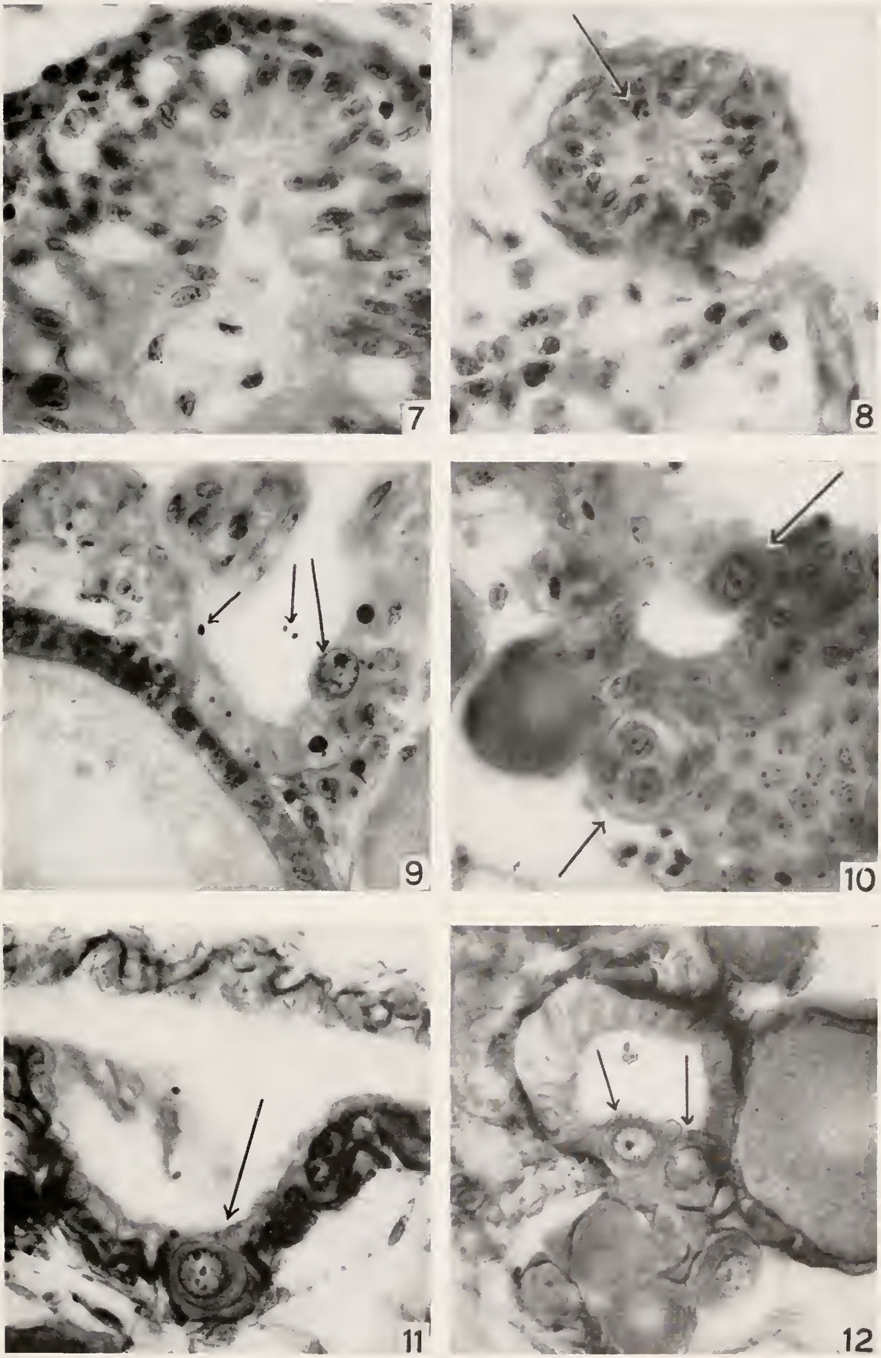

PLATE II[2]

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[2] See footnote 1.

cells normally occur in clusters at the surface of the ovigerous folds (Figures 2, 10, 12). This is true particularly at the interlobular fissures that occur in the ovigerous folds. The attachment of the full grown oöcytes to the ovarian delle has been described in another part of this paper. Histologically, it is evident that the subepithelial connective tissue fibers are in direct continuity with those surrounding the large individual cells and clusters of small cells (Figure 10). Furthermore, there are frequent examples of prominent invaginations of the ovigerous fold epithelium to form small nests or clusters of epithelial cells. These invaginated nests to all purposes appear like clusters of typical gonial cells (Figures 10, 12). In addition, typical growing oöcytes frequently appear in these invaginated cell nests (Figure 12). The striking feature of many of the nests is that the cells still are in direct continuity with the epithelial cells on the surface of the ovigerous fold; in other words, the invaginated cluster of cells has not been pinched off as yet from the superficial epithelium. With differential stains, it is possible to determine that the invagination of the cells does not always break through the underlying, subepithelial connective tissue fibers but rather that the heavy fibers and the accompanying fibrocytes are carried along with the invagination to form a thin connective tissue sheath around the nest of cells. Thus, in these cases, not only the cells within the nests but also the connective tissue fibers around the nests are continuous with the corresponding elements at the surface of the ovigerous folds. These nests then are pinched off from the surface. Lastly, the observation has been made frequently that oöcytes may occur within the epithelium proper of the ovigerous folds. They are much larger than the adjacent epithelial cells and have the customary large vesicular nucleus (Figures 9 to 12). A secondary migration of these oöcytes into the epithelium appears unlikely. Rather, it appears that these are examples of epithelial cells differentiating *in situ* to form germ cells. In these cases, differentiation occurs without the usual invagination. Therefore, in view of (1) the intimate histological connection between the oöcytes and the ovigerous fold epithelium, (2) the actual invagination of the epithelial cells, and (3) the occurrence of typical oöcytes in the epithelium proper, the writer is firmly convinced that, in the adult gonad at least, some of the germ cells arise from the ovarian epithelium on the ovigerous folds. It is interesting that Turner made similar observations in the ovary of *Anableps anableps*. He not only stressed the subepithelial position of the germ cell nests and oöcytes but also noted occasionally single oöcytes in the epithelium itself. However, he arrived at no conclusions regarding the origin of the germ cells.

SUMMARY

1. Two ovigerous folds, one on either side of the median sagittal septum of the ovary are described as bearing the germ cells.
2. Oöcytes normally occur in clusters at the surface of the ovigerous folds.
3. Follicular pores or delles not only facilitate access of the spermatozoa to the ovum but also provide a place for the escape of the fertilized egg.
4. The growing oöcyte is characterized largely by the absence of large masses of yolk and the presence of numerous oil droplets. During growth the nucleus changes from a typical germinal vesicle to a granular, eosinophylic body in which the chromatin exhibits only a weak affinity for stains.

5. The follicle of Neotoca changes during growth from a tenuous layer of scattered squamous cells to a thick, simple layer of columnar cells.

6. Fertilization, the completion of the meiotic divisions, and the escape of the egg are described as occurring simultaneously or in extremely rapid succession.

7. Normally evacuated follicles cannot be compared in any way to the mammalian corpus luteum.

8. In the atresia of the follicles the following phenomena are believed to occur: some fatty degeneration, some liquefaction or vacuolization of cells, some lymphocyte infiltration, some phagocytosis, and lastly, some absorption by the surrounding cells.

9. Some, if not most, of the germ cells of the adult gonad of the female are believed to arise from the epithelium of the ovigerous folds.

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THE REACTION OF CERTAIN CRUSTACEA TO DIRECT AND TO DIFFUSE LIGHT

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Many plankton organisms which move downward, away from daylight in the sea, move toward a source of light in the laboratory. It was suggested in a previous paper (Schallek, 1942) that this behavior is caused by the animals' being exposed to diffuse light in nature, but to direct light in the laboratory. It was reported that when a tall glass cylinder containing the copepod *Acartia tonsa* was illuminated from above, the animals swam up toward the light. This phototropic reaction seemed to depend on the fact that the light was shining directly down the axis of the cylinder. But when the lamp was moved so as to illuminate the container obliquely, the refraction of the light at the surface of the water and its reflection from the curved inner wall of the cylinder seemed to form a diffuse illumination. Under these circumstances the animals sank downward, simulating their behavior in nature. The present paper provides a further development of this hypothesis based on measurement of the light distribution inside the cylinder.

APPARATUS

A glass cylinder 18 inches high and six inches in diameter was used for the experiments. The cylinder was kept at 15° C. in a constant temperature bath 15 inches high and 15 × 18 inches in area. The bath had black walls to absorb reflected light, and was kept in a photographic darkroom. Illumination was from a 75-watt lamp with a parabolic, aluminum-coated reflector. The lamp was placed in five different positions, each 12 inches from the top of the cylinder, but separated by 22.5°. Position 1 was on a level with the top of the cylinder, while position 5 was directly overhead.

Light measurements were made with a Westinghouse "Photox" cell connected to a micro-ammeter, and calibrated in foot candles with a Macbeth illuminometer. The cell was placed in a waterproof case, and covered with a cylindrical hood, limiting the light received to an angular opening of 22.5°. The cell was held six inches below the surface of the cylinder by clamps and rods attached to a ringstand. As this apparatus would not fit inside the six-inch cylinder, an 11-inch cylinder was used for the light measurements. This change is not believed to be a significant source of error.

Twenty *Acartia tonsa* were placed in the cylinder at a time, while the lamp was shifted from position 1 (oblique) to position 5 (overhead). Alternate runs were made in the opposite direction. Two hours' exposure to each position was allowed before counting to permit the animals to reach equilibrium. Results are presented as the percentage of animals in the top third of the cylinder.

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[1] Contribution No. 323.

OBSERVATIONS

A. tonsa moves down when the illumination is oblique, and moves up when the lamp is overhead (Table I). Measurements of the light distribution inside the cylinder are given in Figure 1.

TABLE I

Distribution of animals in cylinder
Average of three runs with 20 animals each

Light position	1	2	3	4	5
Elevation above horizontal	0	22.5	45	67.5	90°
	Per cent animals in top third of cylinder				
Acartia tonsa	13	29	47	65	68
Centropages typicus	69	70	72	72	79

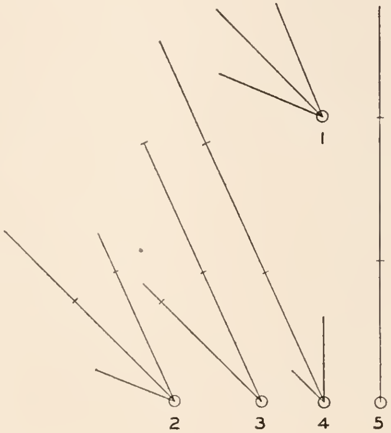

FIGURE 1. Light distribution inside the cylinder for each position of lamp. The small circle indicates the location of the photometer, six inches below the surface of the water. The number of each lamp position is given below the circle. The length of each line represents the light intensity recorded when the photometer was pointed in that direction. Logarithmic scale: the first crossbar = 100 foot-candles, the second = 1000 F. C. The hood limiting the incident light to 22.5° made the cell insensitive to less than 20 F. C.

These measurements reveal several factors that may influence the behavior of *A. tonsa:*

1. There is a 50-fold increase in the *intensity* of the maximum beam as the lamp is raised over the cylinder. It may be that the animals swim up in a bright light and sink down in a dim one. To test this factor, a slide-wire resistance was connected in series with the lamp, permitting the light intensity to be varied while the lamp was held in the overhead position. A 120-fold increase in the intensity of the light had no effect on the distribution of *A. tonsa* (Table IIA).

2. The *direction* of the maximum beam is vertical in position 5, but is more horizontal in the other positions. Perhaps the animals swim toward a vertical light but sink downward in a horizontal one. This possibility was tested by

TABLE II

Effect of changing light intensity

A. Cylinder vertical, light overhead.

Average of three runs with 20 *A. tonsa* each

Light intensity	10	200	1200 F. C.
Per cent animals in top third of cylinder	56	52	54

B. Horizontal trough, illuminated from end.

Light intensity		10	20	200	1300 F. C.
Time to move 10 inches					
Animal	1	130	150	125	130 seconds
	2	15	25	10	15

placing the cylinder on its side. The light from position 5 (shining directly down the axis of the cylinder) formerly was vertical, but now came in a horizontal direction. When the lamp was in position 1 (oblique) the animals sank downward (Figure 2A); when it was moved to position 5 (now horizontal) the animals swam to the end of the cylinder nearest the light (Figure 2B). Hence the animals swim toward the light when it is parallel to the axis of the cylinder, and sink downward when it is oblique, regardless of whether the light comes from a horizontal or a vertical direction.

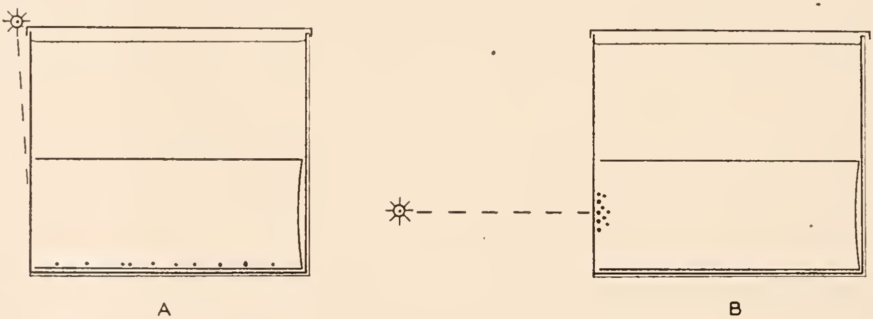

FIGURE 2. Arrangement of apparatus to test effect of light direction. A, lamp in position 1; B, lamp in position 5. Position of animals shown by black dots. The cylinder is on its side in a glass tank painted black (double line) on all sides except one through which the light enters. A piece of tarpaper covers the top.

A further test was made with a horizontal tropism trough, which could be illuminated at either end by a beam of light shining directly down the trough. *A. tonsa* always moved toward the light under these conditions; by turning on first the light at one end and then that at the other, it was possible to keep an individual moving back and forth from end to end indefinitely. Here again the reaction is independent of light intensity (Table IIB).

3. The *distribution* of the light changes as the lamp is moved. In position 5 the illumination is highly directional, with all the light coming from a single direction. In position 1 the illumination is more diffuse, the light coming almost equally from three directions. This angular distribution can be conveniently measured by the ratio (sum of intensities in other directions) : (highest intensity).

The 22.5° hood permitted 16 readings to be taken by rotating the photometer through 360°. For perfectly direct illumination, with all the light coming from one direction, this diffusion ratio will be 0 : 1, or 0. For perfectly diffuse illumination, with the light coming equally from each of the 16 directions in which measurements were made, it will be 15 : 1, or 15. The diffusion ratio for each position of the lamp is given in Table III. The curve formed by plotting these values against the percentage of animals in the top third of the cylinder is shown in Figure 3.

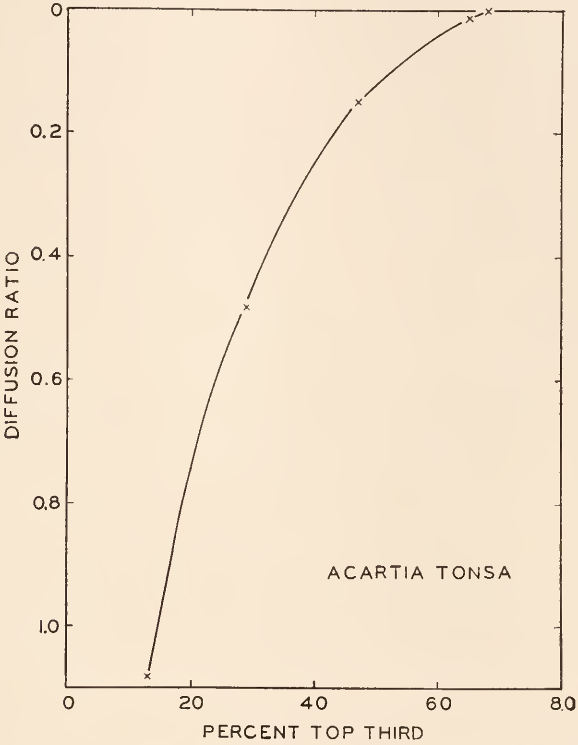

FIGURE 3. Influence of distribution of light on behavior of *A. tonsa*. Ordinate, diffusion ratio of light. Abscissa, per cent of animals in top third of cylinder.

The possibility remains that this relation is an artifact caused by the particular apparatus or procedure used. This is not so, since another copepod, *Centropages typicus*, is only slightly affected by the shift in lighting (Table I). It must therefore be concluded that the behavior of *A. tonsa* depends upon the angular distribution of the light:

1. *A. tonsa* moves toward a source of highly directional light, regardless of the intensity of the light or the direction from which it comes. This is a typical positive phototropism.

2. *A. tonsa* sinks downward in less directional (diffuse) light. This is a positive geotropism and not a negative phototropism, since the animal does not move along the beam of maximum light, but sinks passively downward.

Which of these types of illumination will animals encounter in nature? Measurements were made of light distribution in air, while the distribution of light in the sea was calculated from the data of Whitney (1941) (Figure 4, Table III).

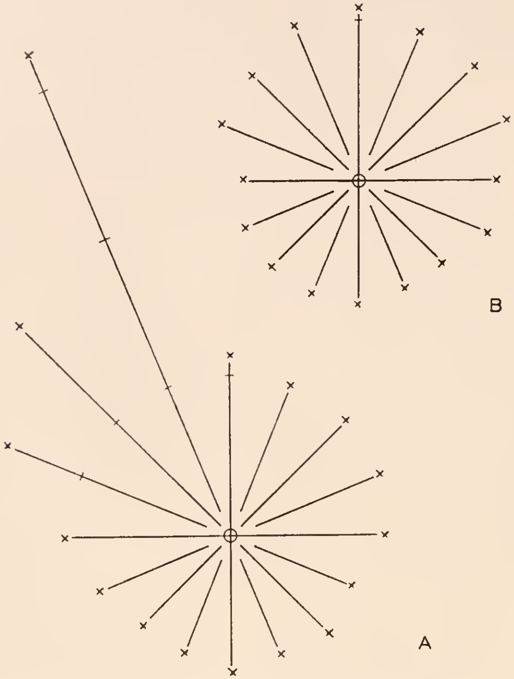

FIGURE 4. Light distribution three feet above roof of Marine Biological Laboratory. August 2, 1942, 10 A.M. Arrangement as in Figure 1.
A. Clear sky, plane of sun.
B. Clear sky, plane perpendicular to sun.

It is evident that light in the sea becomes even more diffuse than any conditions reached in the cylinder. *A. tonsa* sinks downward in diffuse light in the laboratory, and this reaction apparently accounts for the fact that it leaves the surface of the sea in the daytime (Esterly, 1928). *Centropages typicus*, which moved down only slightly in the diffuse light in the cylinder, shows only a slight downward movement in the sea (Clarke, 1934).

DISCUSSION

There are several reports in the literature of animals which react phototropically in a horizontal tube illuminated from the end, but which sink downward in a vertical tube illuminated obliquely (Table IV). Like *A. tonsa*, these animals apparently have different responses to direct and to diffuse light. Most of them show an upward movement in the dark.

The diversity of these animals, ranging from protozoans to arthropods and echinoderms, suggests that a principle of general importance is involved. A

TABLE III

Summary of light measurements

$$\text{Diffusion ratio} = \frac{\text{sum of other intensities}}{\text{highest intensity}}$$

Complete concentration = 0; complete diffusion = 15

		Diffusion ratio
Measurements in cylinder * six inches below surface		
Light position	1	1.08
	2	0.48
	3	0.15
	4	0.012
	5	0
Measurements in sea (Whitney, 1941) 5 met. below surface		
Clear sky, plane of sun		2.6
perpendicular to sun		3.2
Diffuse sky, any plane		2.0
Measurements in air 3 feet above ground		
Clear sky, plane of sun		0.13
perpendicular to sun		9.2
Diffuse sky, plane of sun		6.2
perpendicular to sun		6.8

* As the hood limiting incident light to 22.5° made the photocell insensitive to less than 20 F. C., these values are probably somewhat lower than the true ones.

TABLE IV

Summary of geotropism experiments

Animal	Phototropism in horizontal tube	Geotropism in vertical tube		Author
		In light	In dark	
Corethra plumicornis larva	+	+	−	Harper, 1907
Cyclops albidus	−	+	−	Esterly, 1907
Branchipus serratus	+	+	−	McGinnis, 1911
Daphnia pulex	+	+	−	Dice, 1914
Sagitta bipunctata	+	+	−	Esterly, 1919
Diadema setosum larva; *Paramecium*	Indif.	+	−	Fox, 1925
Holopedium gibberum	+	+	Indif.	Kikuchi, 1938
Hemimysis lamornae	+ or −	+	−	Foxon, 1940
Acartia tonsa	+	+	−	Schallek, 1942

The nauplii of *Balanus perforatus* sink to the bottom of the aquarium when taken from the dark into horizontal light, but swim up when the lamp is moved overhead (Ewald, 1912). Indif. means indifferent.

possible explanation of this effect is provided by Clark (1933). The beetle *Dineutes* moves toward the lamp if placed in a direct beam of light. If a piece of white cardboard is held perpendicular to the beam 300 cm. behind the animal, occasional circus movements appear. These become more frequent as the cardboard is brought closer to the animal, until at 10 cm. they become continuous.

This effect is attributed to the stimulation of additional ommatidia by the light reflected from the cardboard. In concentrated light, the photoreceptor will be stimulated from the front only, and the animal will then react in a typically phototropic fashion. In diffuse light, however, the photoreceptor will be stimulated from both front and side, and a different behavior appears. In the case of *A. tonsa* this results in cessation of activity, since the animal may be observed to sink passively in diffuse light.

Laboratory studies of the light reactions of animals have largely been concerned with phototropic behavior in a direct beam of light. Measurement of the light distribution in the sea shows that it is much more diffuse than in such experimental conditions. The reaction of *A. tonsa* to diffuse light in the laboratory accords with its downward movement in the ocean during the day. Its reaction in the direct light in which experiments on phototropism are usually conducted has no bearing on its behavior in nature.

Such relations may not be confined to this particular copepod. Several reports have been quoted suggesting similar behavior in other aquatic forms. The measurements of light distribution in air show that it is generally diffuse. Perhaps this will solve the riddle of why positively phototropic insects do not fly up to the sun: they may move toward a direct light but behave differently in diffuse light. Phototropism experiments in a direct beam of light need not necessarily apply to the behavior of organisms in nature.

SUMMARY

When the copepod *Acartia tonsa* is placed in a tall glass cylinder illuminated from above, the animal swims upward. When the cylinder is illuminated obliquely, the animal sinks downward.

Measurement of the light distribution inside the cylinder shows that the behavior of *A. tonsa* depends upon the angular distribution of the light. In highly directional illumination, the animal reacts phototropically, and swims toward the light. In less directional (diffuse) illumination, the animal stops swimming and sinks passively downward.

Measurement of the light distribution in the air and in the sea shows that it is generally more diffuse than the conditions in the cylinder. The reaction of *A. tonsa* to diffuse light in the laboratory accords with its downward movement in the ocean during the day. Its reaction in the direct light in which phototropism experiments are usually performed has no bearing on its behavior in nature.

ACKNOWLEDGMENTS

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STUDIES ON THE LIFE HISTORY OF THE MARINE ANNELID *NEREIS VEXILLOSA*

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Introduction

Nereis vexillosa Grube is the most abundant member of the larger annelids of the Pacific northwest fauna. In Puget Sound it attains a size of about 30 cm. but, as will be explained later, some heteronereid individuals may be only about 6 cm. long.

It is characteristically a cold water form occurring intertidally and in shallow water from eastern Siberia, Alaska and southward along the northwest American coast to Santa Barbara, California. Some reports extend its range as far south as San Diego. In the southern range, however, it may have been confused with *Nereis mediator* Chamberlain (Chamberlain, 1919) or with *Neanthes succinea* (Frey and Leuckart) according to Hartman (personal communication). The apparent absence of the specific egg masses, described below, from the southern range lends support to the belief that *N. vexillosa* may not occur in these waters.

The tiny eggs produced by this animal are about 0.2 mm. in diameter; they are spawned in firm, irregular gelatinoid masses, somewhat translucent, and of blue-green, greenish or brownish tints (Figure 1). These colors are most noticeable in the freshly laid eggs owing to their greater compactness prior to absorption of water by the capsular material. The firmness of the masses enables them to withstand a good deal of handling or washing about on the beach by waves without disintegrating. Hence they are often found in good condition on tidal flats where bits of seaweed or other debris collects at the waters edge. They appear never to be found in more than moderate quantities despite the abundance of the species producing them.

In so far as known, there is no other nereid worm that deposits its eggs in this manner. Commonly the eggs of other species of the genus, or of closely related genera are deposited separately in the water or are only lightly agglutinated. The size of masses varies considerably being about one to three inches in diameter, apparently depending upon the size of the female depositing them.

The identity of the egg masses has long been a puzzle to biologists and others alike, and, indeed, the present study was initiated in 1927 at Friday Harbor, Washington, primarily for the purpose of identification. In the course of the investigation, however, important aspects pertaining to the life history of the species have come to light.

It is a pleasure to acknowledge the generous support given this investigation by Director T. G. Thompson, Professor T. Kincaid, and other members of the staff of the University of Washington Oceanographic Laboratories where facilities

were provided for much of the work. My thanks are also due to Dr. Olga Hartman of the Allen Hancock Foundation for identification of the heteronereis worm and for helpful suggestions.

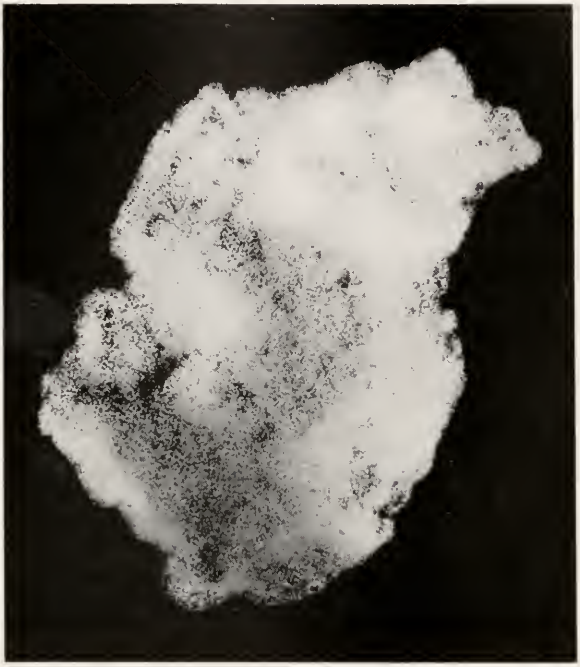


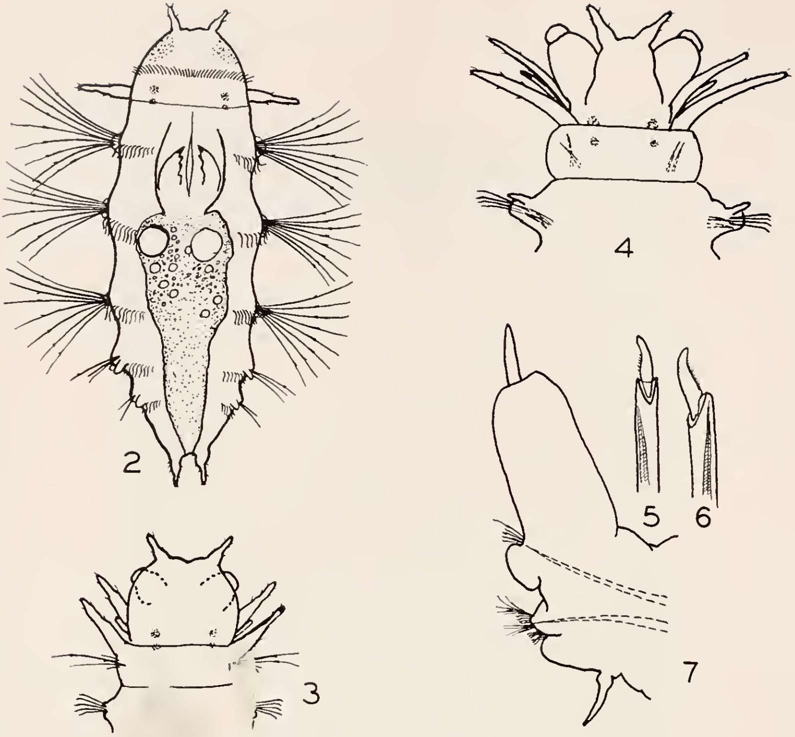
FIGURE 1. *Nereis vexillosa.* A small egg mass, about natural size.

CULTURE EXPERIMENTS AND DEVELOPMENT

The larvae resulting from culture of eggs collected in 1927 failed to survive long beyond hatching and therefore served to establish only that they were annelids of the nereid type. The failure of these to survive may be due in part to inadequate food since they were fed only diatoms and algae. This diet brought good results with *Platynereis agasszi* which was being reared at the same time (see Guberlet, 1933), but subsequent experiments have indicated that *Nereis vexillosa* thrives best in later life when animal food is provided.

In the summer of 1941 an opportunity was again presented to set up cultures at Friday Harbor. Two egg masses were found in False Bay and rearing of the larvae began on June 28. Some of the larvae were already hatching in the three to five segmented stage (Figure 2). The larvae and young worms grew slowly and were able to survive for about a week after hatching on the yolk content which became concentrated in the digestive tract. Later they were fed sessile diatoms (*Navicula sp.* and *Biddulphia laevis*) and powdered dry scallop muscle.

The rate of growth varied so greatly that on August 23 the most advanced worms, now about 57 days old, had acquired 35 segments and were about 9 mm. long, whereas the least advanced had but 15 segments and were about 5 mm. long.



Figures 2–7. *Nereis vexillosa.*

Figure 2. Five-segmented larva.
Figure 3. Head of eight-segmented larva to show development of peristomial tentacles.
Figure 4. Head of fifteen-segmented larva to show further progress of head development.
Figures 5 and 6. Homogomph notoseta and heterogomph neuroseta from parapodium shown in Figure 7.
Figure 7. Parapodium from posterior portion of body of eleven month old worm.
(Camera lucida drawings, Figures 2, 3, 4 same scale; Figures 5, 6, 7 enlarged scale.)

It was however, still not possible to establish identity of the worms from the most advanced specimens, therefore, at the end of the summer session the animals were moved alive to the Scripps Institution of Oceanography at La Jolla, California. This was accomplished by placing ten specimens of various sizes in each of four vials with sea water and containing a wad of cotton among the strands of which the young worms found protection against violent battering in the vials while in transit. The vials were kept cool by being wrapped in wet, frequently-changed paper towels.

Upon arrival at La Jolla the specimens were changed gradually to local sea water (salinity about $3^0/_{00}$ above that at Friday Harbor) in small culture dishes and it was found that 25 had survived the journey. Following some additional mortality in the first month, 16 of various sizes lived for several months.

After setting up the cultures at La Jolla, about three-fourths of the water was changed in each dish every two or three days and the animals were fed dried powdered pecten and sea mussel for a few weeks, but growth was so slow that it was decided to try feeding finely chopped pieces of freshly killed *Thoracophelia mucronata*, a polychaete worm found abundantly at La Jolla. This fresh food was given in small quantities every second or third day. With it there was a prompt response by more rapid growth. Later bits of fresh mussel were also fed but the minced worms seemed to be preferred. Not only the killed and minced worms were eaten but living specimens half the size of the young Nereis were eaten. This rapacious habit was evident even in worms only 6 or 8 mm. long that were seen to grasp and completely swallow in a few seconds others of their kind that were fully half their own size. Diatoms were also added to the dishes and these were readily eaten. The animals rejected partially decomposed food and since the object of the experiment was to keep them growing as long as possible no further experimenting with food was deemed advisable. Results indicate that *N. vexillosa* is more or less omnivorous, utilizing mostly animal food but that it is not a scavenger by preference. According to Copeland and Wieman (1924) *Nereis virens* is also omnivorous though Gross (1921) found only evidence of plant feeding.

No membranous tubes were constructed as in *Platynereis agasszi*, only flimsy tubes of sand and debris were formed with the aid of secreted mucus, and after the animals had reached a length of about 15 mm. they rarely left their tubes completely to gather food. To facilitate study, pieces of glass tubing were provided and these were readily accepted in most cases. Some animals refused to accept new glass tubes that were provided to replace those outgrown and deserted. In these instances the lack of security resulted in restlessness and failure to feed normally.

The older specimens were kept in running (uncooled) sea water pumped in from the sea. At the middle of July this water had reached a temperature of 20° C., which is about 6 to 8 degrees higher than might be expected in the natural habits of *N. vexillosa* near Friday Harbor during the summer. This may well have been a contributing factor in the failure of the worms to survive with the advance of summer at La Jolla.

In Table I is given a summary of the condition of the worms that survived ten or more months. Two of these were found dead (May 18 and May 29). The others were killed and preserved only after they had deserted their tubes and it seemed obvious that they would not survive much longer.

At the age of four and one-half months, when about 60 segments had been formed, the worms reared from the 1941 egg masses had developed specific characteristics of *N. vexillosa*. Especially characteristic are the elongate strap-like dorsal ligules (Figure 7) of the parapodia of the posterior region of the body, but the head structures and the setae of the posterior region are also distinctive (Figures 5 and 6) in older specimens.

The process of cephalization is shown in Figures 2, 3, and 4. In this development, the first setigerous segment of the early larva becomes modified to form the peristomium. The anterior dorsal pair of peristomial tentacles appear first, followed by the posterior dorsal pair which develop from the first larval parapodium. A ventral pair of anterior peristomial tentacles then appear and finally the posterior ventral pair of tentacles are in evidence when the worm has acquired about 18 to 20 segments.

TABLE I

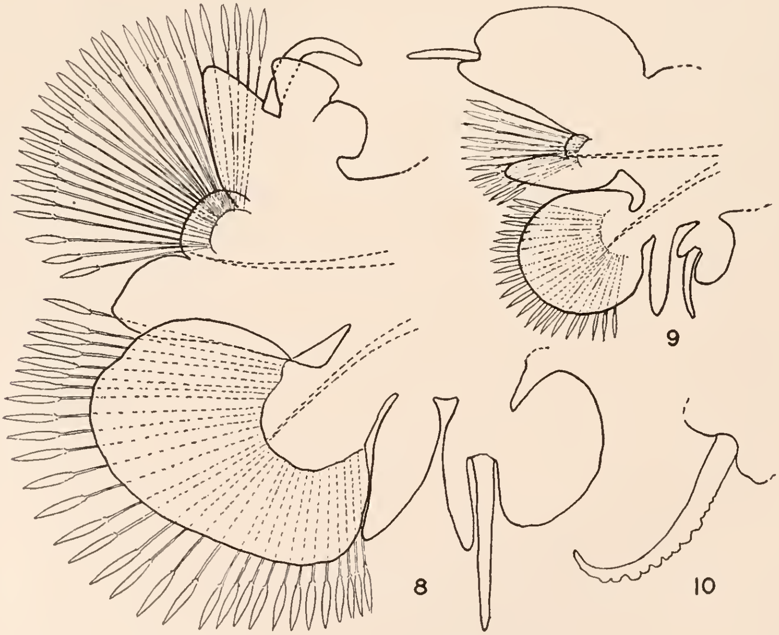
Nereis vexillosa. The seven oldest specimens from cultures set up at Friday Harbor, June 28, 1941

Date killed or found dead 1942	Number of segments	Approximate length when moderately relaxed
April 25	128	8 cm.
May 18	109	5 cm.
May 26	109	6.7 cm.
May 26	107	4.5 cm.
May 29	119	8.5 cm.
June 10	105	7 cm.
July 28	125	10 cm.

None of the specimens showed any indication of entering the heteronereis phase. In this respect they differ from *Platynereis agasszi*, several of which were found to enter this phase near the end of the first year.

SPAWNING HABITS

While culturing the larvae at Friday Harbor in 1941 a watch was kept for spawning adults in the bay. Finally from isolated small, spawning heteronereids there was obtained several masses of eggs. These spawners proved to be *Nereis*



FIGURES 8–10. *Nereis vexillosa.*
FIGURE 8. Heteronereized female parapodium, middle portion of posterior body.
FIGURE 9. Heteronereized female parapodium, fourth from last segment.
FIGURE 10. Dorsal cirrus, heteronereized male parapodium.
(Figures 8, 9, 10 camera lucida drawings to same scale.)

vexillosa Grube thus confirming the identification of the worms reared experimentally from egg masses collected in the field. Heteronereized parapodia are shown in Figures 8 and 9. Heteronereis males have 25 parapodial segments in the anterior portion of the body while the females have 27 such segments (count of 12 males and four females). In the males the dorsal cirrus of the heteronereized segments bears a series of wart-like protuberances on the ventral surface (Figure 10) while in the females examined these cirri were smooth.

The spawning worms were obtained only at night while collecting with a light at the end of the pier in front of the laboratories. They appeared only in small numbers usually an hour or two before midnight and were mingled with spawning swarms of the smaller species *Platynereis* (formerly *Nereis*) *agasszi* which on all occasions was the first of the two to appear swimming at the surface. It was not possible with these few observations to establish any correlation of spawning with phases of the tide or moon as has been done with other marine worms (cf. Woodworth, 1907; Lillie and Just, 1913; Guberlet, 1933).

Only small individuals of *N. vexillosa* were seen spawning and from what has subsequently been learned through the above rearing experiments, it seems certain that these were all spawning for the first time at the age of one year. Not only were some of the experimentally reared worms as large at the age of 10 to 11 months as some of the spawning worms but there was also indication of approach to sexual maturity. One of the worms reared at La Jolla was killed April 25, 1942 after having deserted its tube. Upon examination it was found to contain many eggs which were very small and not yet ripe, but their large numbers might be interpreted to indicate that spawning would normally have occurred in the coming summer. A second worm that died May 29 also showed many eggs. However, it seems certain that in nature some heteronereis individuals must be older than one year at spawning since egg masses of much greater size than those known to have been spawned by the smaller specimens have been found. This is more fully discussed later.

In nature more males than females were observed swimming at the surface. They are the first to come to the nuptial party where, as scattered individuals, they suddenly appear from below and rise to the immediate surface, swimming a few moments there in spirals and loops and then disappearing into the deeper water or the darkness beyond the range of the collector's light. They continue unabated in numbers and vigor as the females a little later appear to join the dance, a dance which seemingly is a climax that marks the ends of their lives, for none of the exhausted individuals kept in captivity was observed to live more than a few days following the act of spawning.

Isolated small heteronereis females ripe with eggs were induced to spawn almost instantly when a few drops of sperm laden water were added to the water in which they were isolated. This is similar to the findings of Lillie and Just (1913) for *Nereis limbata* and of Just (1929) for *Platynereis dumerilii.* In large battery jars the act of spawning by the female *Nereis vexillosa* consists of coming to or near the surface and suddenly exuding a mass of eggs which instantly agglutinates (Figure 11). She then passively sinks to the bottom together with the mass. A few moments later she frees herself from the mass which remains demersal and which in a few hours swells to about three or four times its original size through the absorption of water. The spawning of these demersal masses in water just beyond the low tide limit may account for the finding of fewer egg masses than are commensurate with the number of worms.

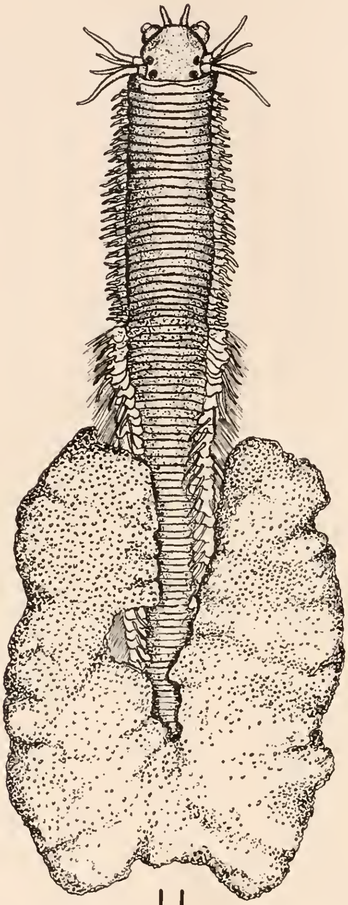


FIGURE 11. *Nereis vexillosa.* Method of egg deposition by small heteronereis. (Free hand drawing.)

DISCUSSION

An answer has been found as to the identity of the egg masses, and certain features of the life history of the species have been discerned. In the process of interpreting the observations, however, another biologically important question arises, namely, does *Nereis vexillosa* possess a diversified life history in which there may be recognized several types of reproductive individuals, as indicated for *Platynereis dumerilii* by Hempelmann? In the latter species at Naples, Hempelmann (1911) distinguished (1) small sexually mature nereis (i.e., atokus) individuals that gave rise to heavily yolked eggs producing characteristic larvae which he called "nereidogene"; (2) small heteronereis forms producing less heavily yolked eggs developing into pelagic larvae called "planktogene"; (3) large heteronereis forms with eggs as in the small form but whose larvae have not been investigated. He found that after spawning, the small nereis form may in experimental cultures be transformed into a small heteronereis form and produce young for the second time and is therefore dissogenous. The nereis form may also grow to a relatively large size and then transform to the large heteronereis but the steps involved in arrival at the large form in this phase are uncertain. It is believed that entrance into the heteronereis form and its spawning marks the end of life for the individual.

More recently Just (1929) also worked on the Naples species and in so far as his investigation was carried, the findings of Hempelmann were verified.

It has been noted by H. P. Johnson (1901) that the heteronereis form of *Nereis vexillosa* occurs in individuals of 56 mm. and upward in length but the maximum length is not given. The same author reports that sexual maturity is frequently arrived at by the species without it becoming heteronereized. Ricketts and Calvin (1939) report finding many large heteronereis of *N. vexillosa* but the authors never found these to be free-swimming. The supposition is, however, that they do spawn, and indeed the finding of egg masses in Puget Sound that are much larger than those known to have been spawned by small heteronereis forms of the species substantiates this.

What the destiny of the worms reared from the Friday Harbor material would have been normally can be only a matter of conjecture. In this connection it may be significant to note that the number of segments in several large (14 to 21 cm. long) *N. vexillosa* collected in the field at Friday Harbor was 142 to 152 in the nereis phase, whereas the spawning heteronereids taken in the same region had only 63 to 96 segments. The latter figure is less than the number occurring in the seven most advanced specimens reared in cultures (Table I). These specimens had 107 to 128 segments and the length of some was greater than the spawning worms. This may mean that the reared worms were destined to reach sexual maturity only in a more advanced nereis phase or in a large heteronereis phase. Much additional study is needed to answer this question. The great range in size that is possible in the heteronereis of *N. vexillosa* should make it an ideal species for such a study if culture problems can be overcome.

SUMMARY

Nereis vexillosa deposits its eggs in firm irregular gelatinoid masses which vary in size from about one to three inches in diameter.

Spawning of small (6 to 8 cm. long) heteronereids of this species was observed to take place an hour or two before midnight. The eggs which are demersal are apparently spawned in water at or just beyond the extreme low tide level and this habit may account for the finding of fewer egg masses than seems commensurate with the number of the species producing them. Isolated heteronereis females were induced to discharge their eggs by the introduction of spermatozoa into the water.

Worms cultured in the laboratory from egg masses collected on the beach throve best on fresh animal food. A number of young worms transported from Friday Harbor to La Jolla attained a maximum length of 10 cm. at the age of 13 months after hatching. All had acquired specific characteristics but none became heteronereized. The transformations taking place during cephalization are similar to that occurring in other nereid worms.

Since the above maximum size of the reared worms is comparable to that of the small spawning heteronereis forms, it appears that the latter were spawning at the age of about one year. Much larger nereis and also heteronereis individuals are known to occur in this species but the time and steps involved in their development are unknown.

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THE REPRODUCTIVE PROCESSES OF THE FISH, ORYZIAS LATIPES

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Introduction

The study of experimental vertebrate embryology has been limited to the breeding seasons of the lower forms with the exception of the amphibia (Rugh, 1941) where ovulation can be induced at almost any time of the year. A study of the reproductive processes of the fish *Oryzias latipes*, the Japanese medaka, was planned in the hope that its eggs might also be made available for more investigations in the experimental field.

Materials and Method

The fish in a sexually mature condition were obtained from a West Coast importer. They were kept in ten-gallon aquaria in a south window, at room temperature and in spring water which was oxygenated by Nitella. The food consisted of freshly collected Tubifex which were cleaned in running water, Daphnia, and some dried fish food. Artifical lighting was supplied only under experimental conditions. This consisted of a 75-watt bulb held above each tank, the light being increased with a metal reflector. When light was to be eliminated this was accomplished by covering the entire tank with a light-tight cardboard box which allowed sufficient circulation of room air and maintenance at room temperature.

The female urino-genital system was studied from cleared whole mounts and serial sections, as well as in the living condition. For a study of the ovulation process females were anesthetized at the appropriate time in MS 222, one part to 3000 of spring water, pinned in a permoplast dish and dissected.

Observations and Experimental Data

The female reproductive system.

The structure of the reproductive tract of the female *Oryzias latipes* (Figures 1–8) was studied as preliminary to observations and experiments on the sexual cycle. The ovary and the oviduct are essentially like those previously described for other poeciliids. The ovary is a median, unpaired sac-like organ (Figures 7 and 8) filling most of the body cavity behind the posterior edges of the liver. It is composed of a large number of follicles in different stages of development, all facing a central lumen which is somewhat occluded by the projecting ripe follicles. These follicles are attached to the thin, muscular ovarian wall on all sides except the dorsal. Through this dorsal wall the lumen and the follicles are visible even in the intact ovary.

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The developing oocytes are practically identical with those of *Fundulus heteroclitus* (Marza, Marza, and Guthrie, 1937; Solberg, 1938), except with respect to size. A small oocyte with a large germinal vesicle becomes transformed into a large egg containing a mass of yolk and oil drops surrounded by a thin layer of cytoplasm. During this growth phase of maturation a thick chorion with long fibers attached to its external surface is laid down around the ovum by the follicle cells. This is a protective outer, non-living membrane comparable to the jelly capsules of the amphibia.

The oviduct is a single thick-walled straight and non-glandular tube about one millimeter in length (Figures 3–8), unpaired as in *Xiphophorus helleri* (Essenberg, 1923; Bailey, 1933; Regnier, 1938) in which species it is not the homologue of the Mullerian duct (Essenberg, 1933). The walls consist largely of circular muscle fibers (Figure 5). The lumen of the oviduct is lined with epithelium which is columnar near the ovary and becomes squamous toward the external opening. Numerous folds in the epithelium of the contracted oviduct allow considerable expansion as the eggs pass rapidly through. There are no glands present, as there are in the amphibian oviduct. The thick muscular walls of the ovary and of the oviduct are histologically continuous, and show no appreciable difference except in regard to total thickness. The duct extends from the posterior end of the ovary to open behind the urino-genital papilla, between the anus and the mesonephric duct (Figure 8). The mesonephric duct descends in the muscle of the body wall posterior to the body cavity to run parallel to the oviduct. Before it does this it bends anteriorly almost to the body cavity and then sharply posteriorly (Figure 8). In the region near the external opening there is no muscular wall, but one is soon organized from the surrounding tissue. In the region of the two loops the lumen is large, the muscular wall is relatively thin, and there are many villi in the mucosa.

The urino-genital papillae are a pair of protuberances from the ventral surface of the female between the anus and oviduct opening (Figure 8). They extend posteriorly and ventrally from their attachment to the body wall, covering the opening of the oviduct, and are grown together in the mid-line for most of their length. The papillae have a thick cortex of stratified epithelium and a highly vascularized medulla.

PLATE I

FIGURE 1. Stained section through external opening of mesonephric duct.
FIGURE 2. Stained section anterior to Figure 1, through mesonephric duct.
FIGURE 3. Stained section anterior to Figure 2, level of mesonephric duct loop.
FIGURE 4. Stained section anterior to Figure 3.
FIGURE 5. Stained section anterior to Figure 4.
FIGURE 6. Stained section through posterior end of body cavity.
FIGURE 7. Stained section through posterior part of ovary.
FIGURE 8. Drawn from a cleared whole mount.

B—body wall; C—connective tissue; D—mesonephric duct; E—edge of rupture; F—fat; I—intestine; L—loop of mesonephric duct; M—muscle; N—capillaries on follicle; O—oviduct; P—body cavity; R—fin ray; S—scale; T—threads on egg; U—urino-genital papilla; V—ovary; X—overgrowth of body wall epithelium; Y—follicle wall; Z—constriction.

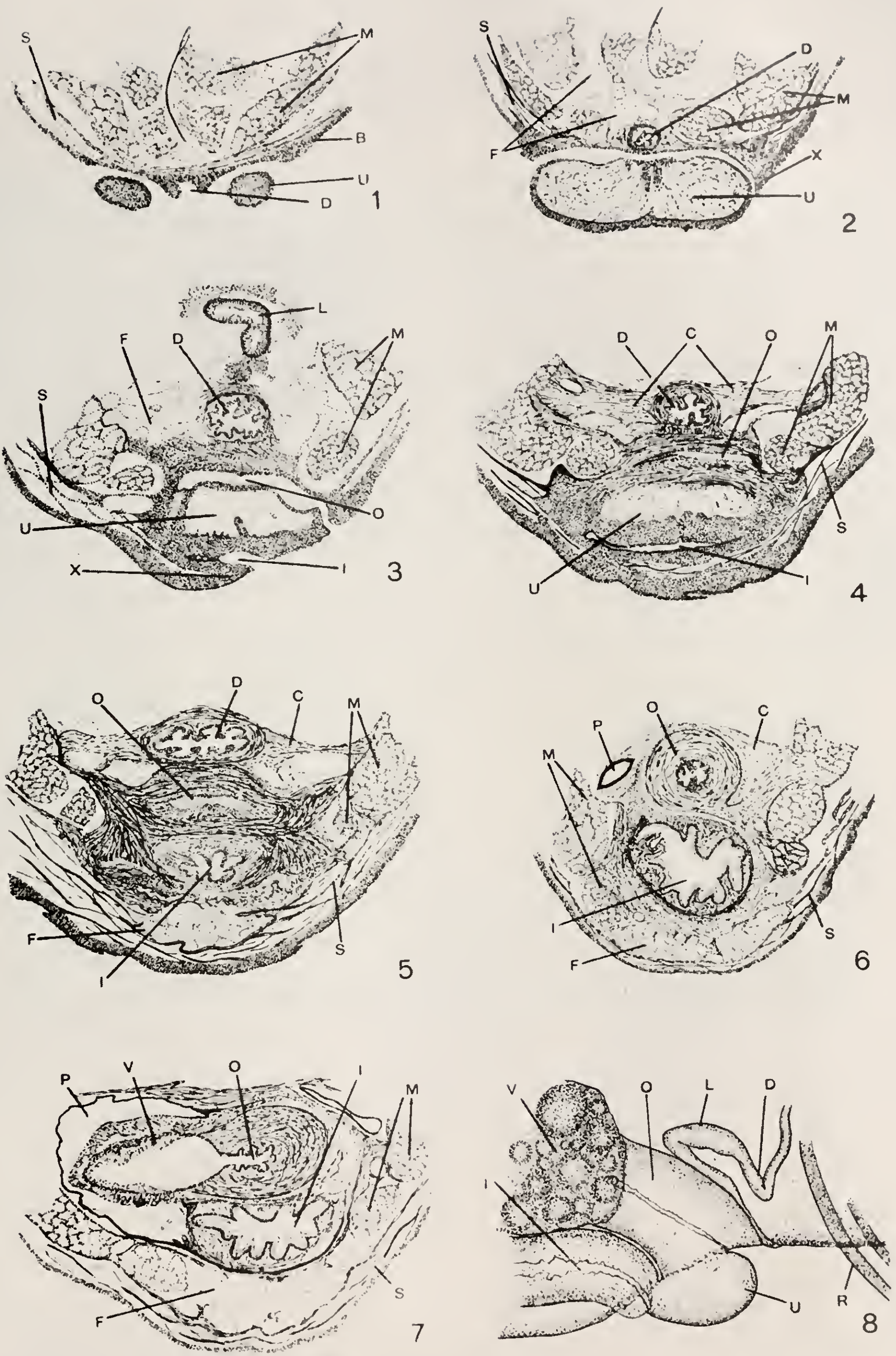
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PLATE I

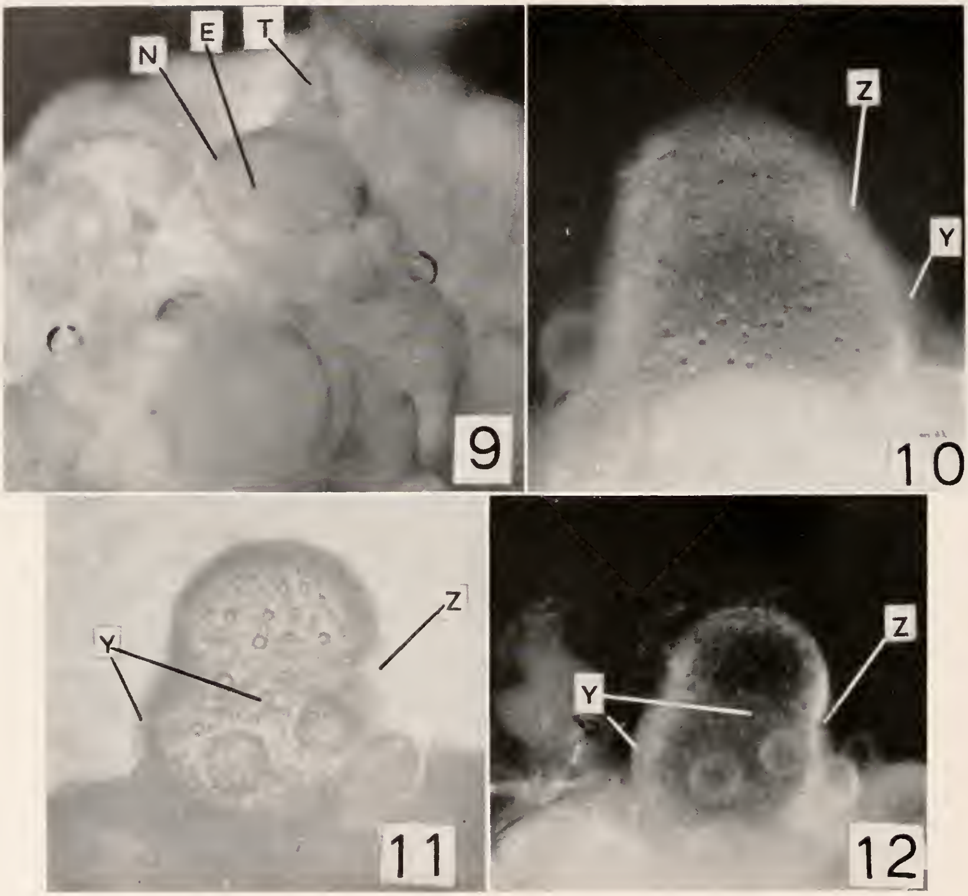

PLATE II

FIGURE 9. Fixed ovary with the dorsal wall removed showing rupture and emergence of egg.
FIGURE 10. Profile view of living egg one-third emerged from follicle.
FIGURE 11. The same egg half emerged, transmitted light.
FIGURE 12. The same, reflected light.

Ovulation and egg transport.

The ovulation process was observed in more than twenty ripe ovaries, either removed from the fish at the appropriate time or observed in partially dissected fish under anesthesia (Figures 9–12). Some of the observations were made directly through the dorsal wall of the intact ovaries, others were made after removal of this dorsal ovarian wall.

The rupture of the ripe follicle appears in the center of a vascular plexus on the side toward the lumen (Figure 9). The hole is generally oval in shape at the beginning of the process, and as it increases in size the blood vessels around it are stretched but do not rupture. As the egg protrudes (Figure 10), the packed threads attached to the chorion become unwound, and the edges of the hole constrict the egg appreciably (Figures 11–12). There is no noticeable increase in the rate of emergence after the egg is half extruded, probably because intraovarian pressure from the muscular walls prevents any sudden eruption. The process of rupture and emergence of the egg from its follicle is very similar to that described for the frog (Rugh, 1935) except that in the frog the egg is released into the body cavity. At the end of the process, from 20 to 30 distorted eggs fill the lumen of the ovary.

The exact duration of the normal process of ovulation cannot be stated since the anesthetized and partially dissected fish are probably not physiologically normal. However, it is possible to estimate approximate times. During these observations most of the eggs in intact ovaries took from 15 to 45 minutes to be extruded. The longer intervals were probably due to the ovary becoming moribund. If intraovarian pressure is removed by tearing the dorsal wall of the ovary, as was done frequently to obtain a better view of individual eggs, an egg may emerge in ten minutes from the time of the first visible rupture. Examining fish taken from an aquarium at intervals during the period of ovulation, it was found that under normal conditions all the fish ovulate completely during a period of about an hour and a half, thus setting that interval as an absolute maximum. Since all fish do not begin to ovulate simultaneously, this estimate is too high for any single specimen and it is presumed that an individual egg normally takes about ten to 20 minutes to emerge from the follicle, and that a single fish ovulates all of its eggs in less than one hour.

The forces causing the initial rupture of the follicle and the extrusion of the egg are unknown. At no time were movements of the ovarian wall or of individual follicles noticeable, although the presence of smooth muscle fibers in both implies the possibility of such movements during ovulation. Furthermore, the whole ovary often undulates remarkably when a fish is first dissected, the contortions resembling euglenoid movement. It is interesting that these movements did not cause rupture of the follicles. Until the forces causing ovulation are known they may be presumed to be similar to those which act in other poikilothermous forms (Rugh, 1935).

The eggs are probably forced into the oviduct by muscular movement of the ovary similar to those observed under the stimulation of dissection. The muscular oviduct and the muscles of the adjacent body wall may also aid in moving the eggs quickly through the short oviduct.

Fertilization.

The mature eggs are not stored in the ovary for more than a few hours, for it was found that normally they are laid almost immediately after ovulation, provided a sufficient number of males is present to supply the incentive. The eggs are laid all at once by the female and are fertilized immediately, while they remain attached to the female by chorionic threads. Egg-laying was not observed, it being a very rapid process, but most of the courtship and fertilization process was observed a number of times. Each time it lasted less than 60 seconds. Kamito (1928) stated that the entire process takes about 35 seconds, and the present observations tend to confirm that statement. After fertilization the eggs are accidentally brushed off on plants, during the day. They remain attached to vegetation during early development.

The maturation cycle.

The maturation cycle of the medaka is 24 hours long and is very regular since the eggs are almost invariably laid in the early morning, although Kamito (1928) reported they may also be laid in the evening. Observation of more than 150 females over a period of three months showed only one possible exception to the rule that the eggs are laid in the early morning. About 75 per cent of all of the adult female fish in the aquarium produce eggs daily. The eggs are laid almost immediately after ovulation. Dissection of numbers of stock fish taken at random at different times of the day never showed ovulated eggs in the ovary before about 1 A.M. or after about 9 A.M. On two occasions six and eight fish were dissected about an hour and a half before the usual time of egg laying and were found to be ovulating or about to ovulate. Thus the time at which the eggs are laid indicates the approximate time of day they were ovulated.

One other important fact was discovered by daily observation of medakas in stock aquaria, although not under experimental conditions. These fish were in aquaria artificially instead of naturally aerated, but otherwise under the same conditions as the other stock and experimental fish. There was no photoperiodicity in these aquaria since lights were above them constantly. These fish did not show the regularity of ovulation that characterizes the species. Newly-laid eggs could be found during any period of the day, and the fish in an aquarium did not lay eggs at approximately the same time, even though the conditions for all were identical.

Experimental Data

The knowledge that the eggs are normally ovulated and laid near dawn, and that unusual lighting conditions upset the regular cycle, suggested that diurnal light changes might be important in controlling the normal cycle. To test this hypothesis experiments were performed as follows:

In the first, eight male and six female specimens of *Oryzias latipes* were put in each of two five-gallon aquaria with food, water, and flora from stock aquaria. One aquarium was covered with a light-tight box from 8:30 P.M. until 8:30 A.M. (Tank I) and the other was covered from 8:30 A.M. until 8:30 P.M. (Tank II).

For the remaining 12 hours each tank was lighted by a 75-watt bulb held directly above in a metal reflector. After one week the lighting schedule for each tank was reversed. The temperature varied with that of the room. The time the eggs were laid was determined either by frequent observation or by noting the stage of cleavage when the eggs were first seen and removed from the female. The developmental rate of the Oryzias egg has been worked out for laboratory temperatures (Rugh, 1941). The first division occurs over an hour after fertilization, and the next two divisions at 45 minute intervals. Results are summarized below (Table I).

TABLE I

Date	Tank I (covered all night)	Tank II (covered all day)
Feb. 2	No eggs all day.	No eggs all day.
3	No eggs all day.	No eggs all day.
4	One laid eggs between 8:30 and 9:30 A.M. No others laid eggs.	One laid eggs between 8:30 and 9:30 P.M. No others laid eggs.
5	One laid eggs between 8:30 and 9:30 A.M. No others laid eggs.	One laid eggs between 8:30 and 9:30 P.M. Others did not lay.
6	One laid eggs between 5 and 8:30 A.M. One laid between 8:30 and 8:40 A.M. No more laid eggs.	Two laid eggs between 5 and 8:30 P.M. One laid eggs between 8:30 and 9 P.M.
7	Two laid eggs between 6 and 8:30 A.M. One laid between 8:30 and 8:40 A.M. No more.	One laid eggs at 9 P.M. One laid eggs between 7 and 8:30 P.M. No more laid.
8	Two laid eggs between 8 and 8:30 A.M. One laid between 8:30 and 9:30 A.M. No more laid.	Tank uncovered at 7:10 P.M. No eggs up to 7:40 P.M.
9	*Schedule reversed. Both tanks uncovered until 2:30 P.M., then tank II was covered.*	
	Tank I	Tank II
10	One with eggs at 2:45 P.M. No other eggs at 8:30 P.M. or at 10 P.M.	No eggs at 8:30 A.M. One laid between 8:30 and 10:30 A.M., another between 10:30 and 11 A.M., another between 11 A.M. and 1 P.M.
11	No eggs at 8:30 A.M., or at 8:30 P.M.	No eggs up to 9 P.M.
12	One laid eggs between 7:30 and 8:20 P.M. Two laid between 8:20 and 10 P.M. No others laid eggs.	No eggs at 8:30 A.M. One laid eggs between 8:30 and 9:50 A.M. No other eggs all day.
13	No eggs at 8:30 P.M. Three laid between 8:30 and 9 P.M. No others laid.	No eggs at 8:30 A.M. One laid between 8:30 A.M. and 1 P.M. No other eggs all day.

The same type of experiment was started with a new set of fish on March 23 (Table II). Twenty females which had been laying eggs between midnight and 8 A.M. without exception for several weeks were put in a ten-gallon aquarium with 22 males, under conditions already described. The aquarium was covered from 8 A.M. until 8 P.M. and lighted with a 75-watt bulb from 8 P.M. until 8 A.M. As controls, six females from the same lot were kept in a community tank and these fish continued to lay eggs nearly every morning. The results appear in the following table:

TABLE II

Date	Observations at 8 P.M.
March 23	No eggs; none laid all day.
24	No eggs; none laid all day.
25	No eggs; none laid all day.
26	Fifteen with eggs, mostly in the two-cell stage, some up to eight cells—i.e., one to three hours old. None laid the rest of the day.
27	Ten with eggs, mostly in the two-cell stage, some up to eight. None laid the rest of the day.
28	Eleven with eggs, mostly in the two-cell stage, some up to eight. None laid the rest of the day.
29	Thirteen with eggs, mostly in the two-cell stage, some up to eight. None laid the rest of the day.
30	Tank covered at 10:30 A.M. and kept covered until 8 P.M. March 31st. Not examined for eggs at 8 P.M.
31	One with newly-laid eggs at 8:30 A.M. Three with eggs in late stages of cleavage at 8 P.M., laid since 8:30 A.M.
April 1	No eggs at 8:40 A.M., nor at 8:40 P.M. Eleven with eggs at 9 P.M. None laid later.

At the end of the experiment the fish were returned to a normal daylight schedule and within two days as many as 75 per cent of the females were laying eggs before 8 A.M. each day.

DISCUSSION

These experiments indicate that the ovulatory cycle of *Oryzias latipes* is in some way correlated with diurnal photoperiodicity. With but four isolated exceptions among the 32 experimental females, reversal of the photoperiodicity changed the time of ovulation, as indicated by egg-laying, by approximately 12 hours. In the first experiment there was an appreciable change in addition to the reversal during the experiment, as the fish had been laying eggs near the middle of the day. In the second experiment two reversals occurred, including the return to normal conditions. The two or three day lag between reversal of the photoperiodicity and appearance of the first eggs is assumed to be caused by an adjustment of the fish to the new lighting conditions.

The exceptional results are easily explained. The two fish in tank II on February 10 undoubtedly were not yet adjusted to the sudden change in photoperiodicity; the one in tank II on February 13 was in poor condition, having been taken from slightly foul water. The exceptional fish on March 31 was the result of the upset of photoperiodicity on the previous day.

These facts suggest the manner in which photoperiodicity may regulate the ovulatory cycle in *Oryzias latipes*. The species is physiologically and genetically adjusted to a daily cycle. This means that 20 to 30 relatively large eggs must be matured, ovulated, and laid in the space of 24 hours, the bulk of this time being utilized in the maturation process. Ovulation (i.e., rupture and emergence of the egg from the ovary) must normally occur approximately at dawn because these studies show that oviposition follows shortly thereafter and *Oryzias latipes* has long been known to produce its eggs early in the morning. It is inconceivable that the temperature of a 15-gallon tank of water would change as abruptly as does the light factor early in the morning. Of the two variables, it seems most likely that since the temperature is relatively constant and the light factor is extremely variable, that this latter factor is the trigger which sets off the ovulation and oviposition reactions. Light in itself may not be necessary in the sense that it acts through the sense organs to bring about breeding reactions for these fish may ovulate on occasion and deposit their eggs even in total darkness, although such reactions are not predictable. It is the thesis of this paper that the stimulus of light brings about an increase in the metabolic activity of the fish and that this activity in turn sets off the breeding reactions which have otherwise been rather quiescent. By direct observation it can be shown that the periods of light and dark do regulate the physical activity of the fish and those which have been in darkness for a considerable period fail to respond normally to tactile stimuli and generally swim very sluggishly. This has been previously reported by Spencer (1929) who found the sunfish to be quiescent at night, and Shaw, Escobar, and Baldwin (1938) who found the locomotor activity of goldfish to be much reduced in reduced sunlight. The medaka is a very active fish during the day so there may be a considerable difference in the amount of energy available for the maturation process between periods of rest (in darkness) and of activity (in light). There is no suggestion that radiant energy is thereby transformed into metabolic energy but rather that light which penetrates the water stimulates the fish to activity which activity in turn utilizes stored metabolic energy. This energy might otherwise be utilized in the maturation process.

Such a thesis does not preclude the possibility of maturation (and ovulation) in fish which are continuously active but that the reproductive cycle of such fish should be longer and more irregular. These observations confirm such a proposition. But under the normal ration of light and darkness the fish will ovulate near the end of the period of darkness no matter what time of day this happens to be. Further confirmation of this thesis might be obtained by enucleation of the females prior to a series of observations, but we would have to go further and attempt to control the chromatophores as well. This would be extremely difficult.

Other species studied have longer cycles, so no direct comparison is possible. It is not impossible that light may also affect these longer cycles, particularly if it can be proven that certain parts of the spectrum are more beneficial than others, such as ultra-violet or the other extreme, infra-red. But this suggested relationship is not offered as the explanation of the phenomena in Oryzias since it produces eggs at almost all seasons. Barney and Anson (1921) and Turner (1936, 1937, 1938) noted a direct relation between seasons and the reproductive cycles in several poeciliids. But these authors associated the breeding reactions more with seasonal temperature than light changes. Dildine (1936) did not agree with Turner's (1937) conclusions after studying *Lebistes reticulatus*. Craig-Bennett (1930) found no effect of variation of light on the cycle of *Gasterosteus aculeatus*.

Matthews (1939) and Burger (1939b), both using the male Fundulus, state that light is not essential and that it has no effect on the spermatogenetic cycle but that temperature is the all-important environmental variable. Matthews showed that low temperatures retarded spermatogenesis and Burger stated that a low light ration of one and a half hours per day kept the fish sexually inactive at 6–10° C., but that many spermatazoa were developed with the same light ration at 14–20° C. It is logical to assume that the low temperature would retard any biological process and Burger admits that a basic light ration of one and a half hours per day is needed to keep the fish sexually active.

Although other environmental factors may be involved, under normal circumstances the time of ovulation (hence also the prior period of maturation) in *Oryzias latipes* is related to the diurnal periodicity, probably through the regulation of the states of activity during the daily cycle rather than through any intrinsic value in light (energy) itself.

Summary and Conclusions

1. The female urino-genital tract of *Oryzias latipes* is, in general, similar to that of other poeciliid fish. The ovary is an unpaired, hollow organ consisting of a thin but strong wall, lined with developing follicles on all sides except the dorsal. The development of the oocytes is similar to that in *Fundulus heteroclitus*. The oviduct is a short, muscular tube with no known function except that of egg transport.

2. The ovulation process is not cataclysmic, but takes an average of about 30 minutes under the conditions stated. Contraction of the ovary as a whole is not necessary, although this does not preclude the possibility of action of smooth muscle in the walls of the follicles themselves. The force causing the initial rupture of the follicle is unknown.

3. The eggs are laid almost immediately after ovulation, either just before or during copulation. Copulation consists of a definite series of actions by both fishes, and the sperm and eggs are shed simultaneously.

4. The fishes normally ovulate just before dawn, and inverting the periods of light and darkness as compared to natural conditions causes ovulation to be shifted from the time of the natural dawn to the time of the artificial dawn. It was suggested that light governs the time of ovulation by regulating the general metabolic activity of the female. The eggs are matured during a period of quiescence and are released at the beginning of the period of activity, stimulated by light. In the normal daily cycle light is probably the most important environmental factor, acting as a stimulant to general activity and hence to ovulation and oviposition in *Oryzias latipes*.

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Bulletin Biologique de la France et de la Belgique (19–50 *as* Bulletin Scientifique de la France et de la Belgique; 1–18 *under various titles beginning* Bulletin Scientifique. . . .) 1869: 1+ **Supplements** 1919: 1+

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Bulletin de la Société Française de Microscopie 1932: 1+

Bulletin de la Société Géophysique de Varsovie *see* Biuletyn Towarzystwa Geofizykow w Warszawie

Bulletin de la Société Linnéenne de Normandie 1855: Caen. 1+

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Bulletin de Pharmacie et des Sciences Accessoires *see* Journal de Pharmacie et de Chimie

Bulletin décadaire (mensuell) Météorologique et Glacial du Service des Prévisions du Temps de l'Administration Centrale des Voies Maritimes du Nord *see* Dekadnyi i Ezhemesi͡achnyi Bi͡ulleten Sluzhby Pogody i Ledovoi Informatsii

Bulletin des Institutions Royales d'Histoire Naturelle à Sophia *see* Izvestii͡a na Tsarskitye Prirodonauchni Instituti v Sofii͡a

***Bulletin des Séances de la Société Belge de Microscopie (Procès-Verbaux)** 1875: 1–25 (*bound in with* Annales de la Société)

Bulletin des Travaux publiés par la Station d'Aquiculture et de Pêche de Castiglione 1926: [1928–33]

Bulletin du Jardin Botanique de Buitenzorg *see* Bulletin of the Botanic Gardens; Buitenzorg

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Bulletin du Jardin Botanique de l'Académie des Sciences de l'URSS (Principal de l'URSS) *see* Izvestii͡a Botanicheskogo Sada

Bulletin du Jardin Botanique de l'État à Bruxelles; Ministère de l'Agriculture 1902: 1, fasc. 4+

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nationale de Plasmogénie (1–88 *as* Bulletin du Laboratoire de Plasmogénie) 1932: Mexico. 1+

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Bulletin(s) et Mémoires de la Société de Neurologie, Psychiatrie et Psychologie de Jassy *see* Bulletin de la Société Roumaine de Neurologie, Psychiatrie, Psychologie et Endocrinologie

*__Bulletin for Scientific Relations; International Institute of Intellectual Co-operation; League of Nations__ 1926: Paris. 1–3

Bulletin from the Laboratories of Natural History of the State University of Iowa *see* University of Iowa Studies in Natural History

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Bulletin Mensuel de la Société Linnéenne de Lyon 1932: [1–10]

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Bulletin(s) of Ichthyological Laboratory of Baku *see* Izvestii͡a Bakinskoi Ikhtiologicheskoi Laboratorii

Bulletin of Mathematical Biophysics (1 published as supplement to Psychometrika) 1939: 1+

Bulletin of Plant Protection (Trudy po Zashchite Rastenii); Lenin Academy of Agricultural Sciences in U.S.S.R.; Institute for Plant Protection 1930: **series 1. Entomology:** vol. 1, nos. 1–2 (1930–31); vol. 3, no. 1 (1931); vol. 4, no. 1 (1931), nos. 3 (1932), 4 (1932), 6 (1932); **series 2. Phytopathology:** no. 3 (1933)

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Bulletin of the Academy of Sciences of the United Provinces of Agra and Oudh Allahabad *see* Proceedings of the National Academy of Sciences; India

Bulletin of the American Association of University Professors 1915: 1+

Bulletin of the American Meteorological Society 1920: 1+

Bulletin of the American Museum of Natural History 1881: 1–19; 23+

*__Bulletin of the Antivenin Institute of America__ 1927: 1–5, no. 3

Bulletin of the Arctic Institute of the USSR *see* Bi͡ulleten Arkticheskogo Instituta

Bulletin of the Association of American Medical Colleges *see* Journal of the Association of American Medical Colleges

Bulletin of the Auckland Institute and Museum 1941: New Zealand. 1+

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***Bulletin of the Brookville Society of Natural History;** contributions to the natural history of Franklin County, Indiana 1885: 1–2

***Bulletin of the Buffalo Naturalists' Field Club** 1883: 1

Bulletin of the Buffalo Society of Natural Sciences 1873: 1+

Bulletin of the Bureau of Agricultural Microbiology, Leningrad *see* Trudy Vsesoi͡uznogo Instituta sel'skokhozi͡aistvennoi Mikrobiologii

Bulletin of the Bureau of Eugenics *see* Trudy Instituta Genetiki

Bulletin of the Bureau of Standards *see* Scientific Papers of the Bureau of Standards

Bulletin of the Bussey Institution; Harvard University 1874: 1–3, pt. 4

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Bulletin(s) of the Ceylon Fisheries *see* Ceylon Journal of Science; Section C. Fisheries

Bulletin of the Chemical Society of Japan 1926: 1+

Bulletin of the Chicago Academy of Science 1883: 1; [2]; 3; [4–5]

***Bulletin of the College of Agriculture, Tōkyō Imperial University** 1887: 5–6

***Bulletin of the Department of Biology of Yenching University** 1930: Peiping. 1

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Bulletin of the Fan Memorial Institute of Biology (5+ in two sections; Botany and Zoology) 1929: 1+

Bulletin of the Fishery Experiment Station, Canton, China 1929: 1929–30 (text in Chinese)

Bulletin of the Fishery Experiment Station of the Government-General of Chosen 1925: Fusan. 1+

Bulletin of the Geographical Society of Philadelphia 1893: 3, no. 4 (1902)

Bulletin of the Geological Institution of the University of Upsala 1892: 1–5; 8; 10–11; [13]; 14–16; 18

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Bulletin of the Harpswell Laboratory *see* Bulletin of the Mount Desert Island Biological Laboratory

Bulletin of the History of Medicine; organ of the American Association of the History of Medicine and the Johns Hopkins Institute of the History of Medicine (1–6 as Bulletin of the Institute of the History of Medicine; 1–2 a supplement to Bulletin of the Johns Hopkins Hospital) 1933: 1+

Bulletin of the Hydrographic Department, Imperial Japanese Navy 1917: 1+

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***Bulletin of the Illinois State Museum of Natural History** 1876: 1; 3–10

Bulletin of the Illinois State Natural History Survey *see* Illinois Natural History Survey Bulletin

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Bulletin of the Institute of Jamaica; Science Series 1940: 1+

Bulletin of the Institute of Physical and Chemical Research (Rikwagaku-Kenkyujo Iho) *see with* Scientific Papers of the Institute of Physical and Chemical Research, Tokyo

Bulletin of the Japanese Society of Scientific Fisheries 1932: 1+

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Bulletin(s) of the Laboratory of Genetics (Trudy Laboratorii Genetiki) *see* Trudy Instituta Genetiki

Bulletin of the Lloyd Library of Botany, Pharmacy and Materia Medica 1900: 1+

Bulletin of the Madras Government Museum 1894: **n.s. Natural History Section** 1927: 1+

***Bulletin of the Michigan Fish Commission** 1890: 1–8

***Bulletin of the Minnesota Academy of Natural Sciences** 1873: [2–4]

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Bulletin of the Museum of Comparative Zoology at Harvard College in Cambridge 1863: 1–2; [3]; 4–33; 35+

Bulletin of the National Research Council (nos. 1–57 *also as* vols. 1–11) 1919: 1+

***Bulletin of the Natural History Society of British Columbia** 1893: 1

Bulletin of the Natural History Society of New Brunswick 1882: 1–2; 4–9; 11–18; 20–21

Bulletin of the Natural History Survey; Chicago Academy of Sciences 1896: 3; 5–6; 7, no. 2; 8

***Bulletin of the Neurological Institute of New York** 1931: 1–7

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***Bulletin of the Philosophical Society of Washington** 1871: 1–15

Bulletin of the Scientific Laboratories of Denison University 1885: 1–12; [13]; 14–17

Bulletin of the Scripps Institution of Oceanography (1–11 as Bulletin of the Scripps Institution for Biological Research); **Non-technical series** 1916: 1+; **technical series** 1927: 1+

Bulletin of the Shanghai Science Institute 1929: 1+

***Bulletin of the South African Biological Society** 1918: Pretoria. ser. 1, no. 2

Bulletin of the State Oceanographical Institute *see* Bi͡ulleten Gosudarstvennogo Okeanograficheskogo Instituta

Bulletin of the Torrey Botanical Club 1870: 1+

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THE FUNCTION OF THE CORPUS ALLATUM IN MUSCOID DIPTERA

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INTRODUCTION

Since the original work of Wigglesworth (1926) it has become increasingly obvious that the corpora allata may exert an influence on the reproductive processes of insects (see Scharrer, 1941, for review). Although Thomsen (1940) concludes that in the adult muscid flies the single median corpus allatum controls the ripening of the ovaries, it is not yet established whether this action is a direct one, and whether there is a true sex hormone produced by the corpus allatum. The fact that Thomsen (l.c.) was able to induce hypertrophy of the corpus allatum by ovariectomy suggests a direct hormonal action, but cannot be taken as proof. In an attempt to throw further light on this problem the experiments to be described were performed. They are concerned with the role of the ring gland in the adult fly, and while the problem is as yet unsettled, considerable information has been gained and a tentative conclusion may be reached. The significance of the work lies in the fact that it bears finally on a major biological problem—the mode of action of hormones on cells and tissues.

MATERIALS AND METHODS

Two species of Muscidae have been employed. A pure strain of *Lucilia sericata* Meig. maintained by mass inbreeding for over 230 generations was used in all earlier work. Search for a more robust fly resulted in the use of a stock of *Sarcophaga securifera* Villeneuve obtained from eggs deposited on meat, in the fall of 1941 in St. Louis, Missouri. Since that time the stock has been maintained by mass inbreeding in the laboratory.

The experiments have consisted mainly of extirpations and implantations of adult organs. Operations were performed under a magnification of 30 diameters and the essential instruments were No. 12 hard steel needles, appropriately sharpened on a hard Arkansas oilstone, and fine iridectomy forceps. Flies were etherized and held in "Permoplast" with cross pins. The corpus allatum may be removed through the neck region if the head of the fly is bent forward; gonads are removed by making a long transverse incision in the intersegmental membrane between the 4th and 5th abdominal segments. With practice the mortality can be reduced to very low figures for both operations, but I have never succeeded in performing both extirpations on a single fly, even when a day elapsed between the experiments.

In the experiments involving the transplantation of the ring gland, portions of the oesophagus invariably had to be included with the transplant, for the ring gland alone was too small to be moved without injury. The site of implantation was under the dorsal abdominal wall between the 2nd and 3rd scuta. In later experiments, in an effort to induce innervation of the transplant, attempts were made to place the ring gland near the brain, which was slightly injured in order to stimulate the growth of nerves to the implant. These attempts were unsuccessful.

A total of 73 successful operations were performed on Lucilia and 102 on Sarcophaga.

At the conclusion of all experiments the flies were fixed by injection with alcoholic Bouin's, and 10 micron paraffin sections were stained either in Mallory's triple stain or by Bodian's protargol technique. Results with both species of flies were similar, unless the contrary is stated.

Description of the Normal Histology

The histological effects of extirpation of the ring gland have not previously been described. In order that they may be more easily followed, a brief description of the normal histology is necessary.

A. *The Ring Gland.* The ring gland of *Lucilia* has been shown (Day, 1942) to be composed of a single median corpus allatum fused with the corpora cardiaca and the hypocerebral ganglion. The situation is similar in Calliphora (Thomsen, 1941) and in Sarcophaga (Figures 3, 4, and 5). Thomsen (1941) refers briefly to the larger size of the corpus allatum in mature flies compared with newly emerged flies (compare her Figures 3 and 4). The changes occurring during the first seven days after emergence of adult *Lucilia sericata* and from emergence to 20 days of age of Sarcophaga, have been carefully followed. The most striking changes occur in the first five days and thereafter there is not much alteration

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Plate I

Corpora allata and fat body of *Lucilia sericata* and *Sarcophaga securifera* fixed in alcoholic Bouin, Bodian protargol method. Photomicrographs, magnification 400 diameters.

1. Corpus allatum of normal female *L. sericata*, transverse section. Note larger nuclei on periphery of the gland, and below a little striated muscle from the dorsal vessel. Cell walls are not easily seen but are present.

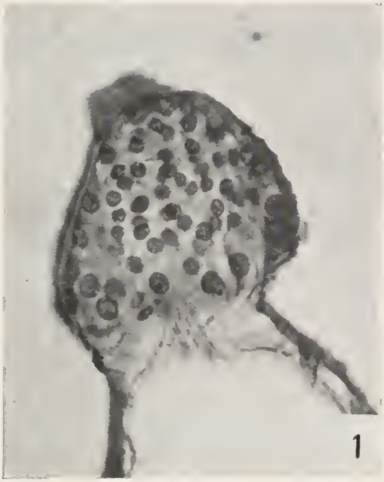
2. The same of a female castrated seven days. Note the hypertrophy of the cells and nuclei. The gland approximately 50 per cent greater in diameter than the control even though there are fewer cells in the section. The hypertrophy of the nuclei is particularly striking and is shown by the larger peripheral ones as well as the central ones. Cell walls are clearly seen.

3. Corpus allatum of *S. securifera* in which recurrent nerve was cut seven days earlier. Note nerve fibers ramifying between cells. In comparison with Figures 4 and 5 note the hypertrophy of cells and nuclei.

4. Corpus allatum of male castrate *S. securifera*.

5. Corpus allatum of female castrate *S. securifera*. Note that there is no hypertrophy in either this gland or that in Figure 4 comparable with that found in Lucilia.

6. Fat body of female *Sarcophaga securifera* showing darkly stained oenocytes. Tissues are essentially normal. Compare with Figure 7.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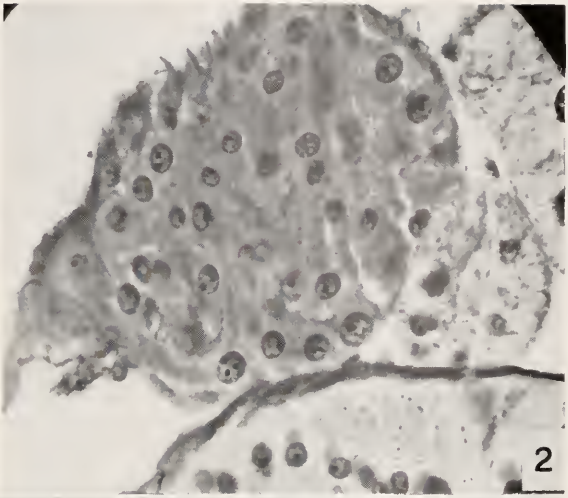
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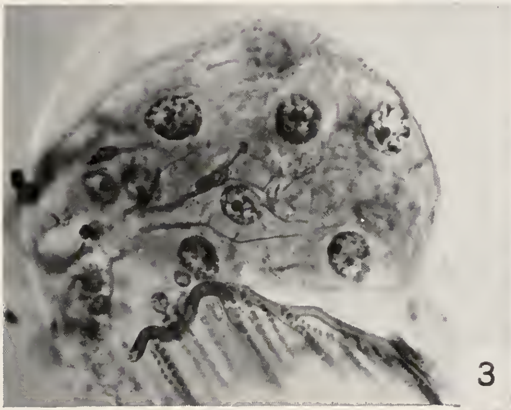
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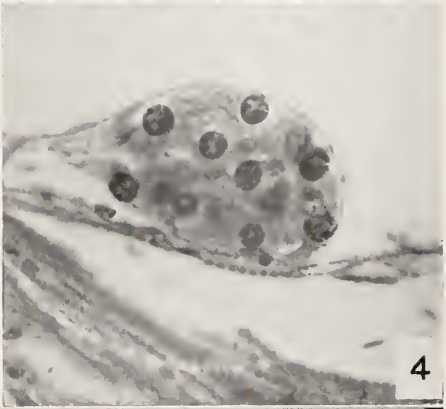
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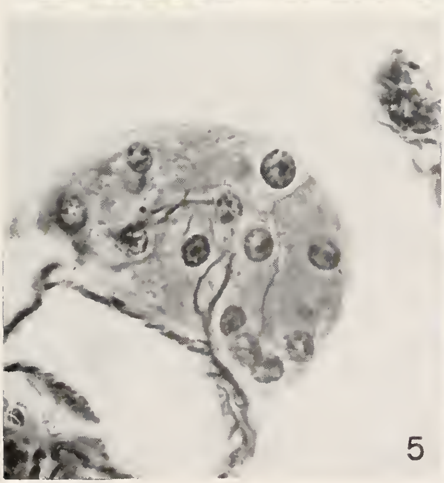
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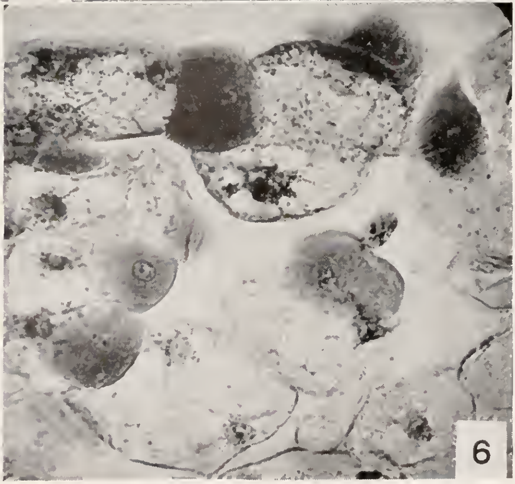
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PLATE I

in the cytology of the gland. Differences between the sexes are insignificant. In Sarcophaga all the fuchsinophilic droplets in the corpus cardiacum cells have disappeared at the time of emergence, but persist for about three days in Lucilia (Day, 1942). In Lucilia larger nuclei seem to occur on the periphery of the gland, but this is not so marked in Sarcophaga. In both there is a thin sheath, which has small flattened nuclei, surrounding the gland. The innervation of the gland is well shown in Bodian preparations and, as in other insects, is very profuse (see especially Figures 3 and 4). Nerves from the hypocerebral ganglion run dorsally in the lateral walls of the aorta and ramify between almost every cell of the corpus allatum. It is almost certain that fine nerves penetrate cells and end near the nucleus in small terminal swellings. Cell membranes are not clearly seen in the illustrations, but the gland is not syncytial. It is well tracheated, but no specializations for transferring secretory products to the aorta are found.

B. *The Fat Body.* As will be shown later the fat body undergoes striking changes after allatectomy. Its normal histology is therefore discussed here and considerable attention has been paid to this tissue in all experimental animals. Teunissen (1937) and Pérez (1918) have described the changes in the fat body of flies during the pupal period, but no adequate description is to be found of subsequent changes. Evans (1935) has described the fat body of adult Lucilia and Roubaud (1932) has described chemical changes in that of *Culex pipiens.* In Sarcophaga I have found that larval fat body cells persist in normal flies for three days after emergence (Figure 7). Their fuchsinophilic contents gradually diminish until the cytoplasm becomes clear and reduced, though the large nucleus still makes it easy to distinguish the cells. The adult fat body cells are at first small but during the first three days of adult life they gradually increase in size. It should be noted that fat body cells differ in various parts of the body. This discussion is limited to the fat body of the lateral body wall in the segments containing the gonads. No substantial difference was observed between the sexes.

Young cells show most frequently four nuclei, but may possess more. A count of the number of nuclei in fat body cells of Sarcophaga gave the following results: Two nuclei were found in 15 per cent of the cells, four in 50 per cent, six in 5 per cent and eight in 30 per cent. The counts were made from acetic orcein spreads from which counts may be made easily and accurately, while this is not possible from sections. No significant change was found between young and old flies, nor is there a difference between the sexes. There is, however, a marked difference depending on where the fat body is located. Cells near the heart are smaller and have fewer nuclei than those located on the lateral body wall. When the fat body is abundant the cells composing it are closely appressed and their exact connections are impossible to determine. In living spread preparations, especially of older flies in which the fat body is less abundant, it is found that the fat body cells are arranged in cords which branch and in which the oenocytes lie in between almost every two cells. In young cells the nuclei are spherical and possess a single, usually slightly eccentric nucleolus. The cytoplasm is aggregated around the periphery of the cell. Within 24 hours after emergence the cells have increased considerably in size, the nuclei have become more nearly centrally located and the nuclear membrane has become slightly irregular in outline and their nucleoli have enlarged considerably. The cytoplasm is uniform, but is lightly aggregated around the nuclei. If the flies continue on a carbohydrate diet no further change occurs. A protein meal, however, changes the appearance of the cytoplasm considerably. It becomes more abundant and thick strands run from the cell wall to the nuclei. The nuclei frequently regain their spherical shape.

In Lucilia, the changes undergone by the fat body are essentially similar to those just described (see Evans, 1935).

C. *The Oenocytes.* Intimately associated with the fat body are the oenocytes. Snodgrass (1935, p. 411) made the undocumented statement that "oenocytes are not known to occur in adult Diptera." However, oenocytes in adult Diptera have been described by Pérez (1910) in Calliphora and by Evans (1935) in Lucilia.

In the newly-emerged Lucilia they are not striking, but with the increase in size of the fat body cells the oenocytes become more conspicuous. They are characterized by uniform, basophilic cytoplasm. Many of the cells, which are uniformly scattered among the abdominal fat body cells, are uninucleate, but about 50 per cent are binucleate. More rarely three or four nuclei are found. Nuclear size in Lucilia oenocytes with a single nucleus averages about six microns, but is less when there are more nuclei per cell. It was early noticed that there was a marked and constant difference between the oenocytes of the two sexes of Lucilia. While those of the male were large and well filled with cytoplasm so that the cell boundaries were convex, those of the females were less conspicuous and had concave cell boundaries. This difference could be clearly seen in the living fat body if the fly was injected with a solution of methylene blue which stains oenocytes specifically. When a similar condition was found in such widely separated flies as *Melophagus ovinus* L. and *Culex pipiens* L., it was thought that the situation might be general in the Diptera. It was so striking that it seemed surprising that no record of this could be found in the literature. It was therefore unexpected to find that *Sarcophaga securifera* did not conform in this respect, the oenocytes being if anything more conspicuous in the female than in the male, although there was little difference between the sexes. The explanation for this is quite unknown but is obviously significant in the explanation of the sexual differences, which must lie eventually in a knowledge of the function of the oenocytes. Without diverging unnecessarily it seems that the majority of evidence points to their functioning as organs of intermediary metabolism (see Wigglesworth, 1939, p. 244). The validity of the interpretation of the following results rests in part on the very plausible assumption that this is at least one of their functions.

In normal flies the oenocytes are very constant, and no cytological evidence of secretory changes can be seen in the adult, though there are some indications that they show an inverse size relationship with the cells of the fat body, and vary slightly with the nutritional state of the insect. As will be shown below, they undergo marked changes upon extirpation of the ring gland.

D. *The Ovaries.* For purposes of subsequent descriptions it is necessary to review briefly the growth of the eggs in the ovary of Lucilia. The relation between nutrition and egg production of muscids has been discussed by Glaser (1923) and Mackerras (1933), and the development of the ovaries of Anopheles by Nicholson (1921). Nicholson divided the growth of the oöcyte into two stages. The first of these represents the growth up to a resting stage in which the oöcytes remain until the insect has taken a protein meal (see Trager, 1941,

p. 23). Histologically such oöcytes are easily distinguished, for no yolk has yet been laid down in them. In Sarcophaga the follicle attains a maximum diameter of about 160 microns. Immediately after feeding on meat, yolk is deposited and the oöcyte increases in size to about three times the size reached in stage I. The nurse cells undergo little change. Once yolk deposition has begun the follicles increase rapidly in size. The follicular cells increase in number but decrease in size. They change from cuboidal towards squamous cells. Later the chorion is laid down.

THE EFFECTS OF EXTIRPATION OF THE RING GLAND

As mentioned above, extirpation of the ring gland of adult flies can be performed with comparative ease. It is, however, not possible to determine without histological examination whether both corpora allata and cardiaca are removed. Thomsen records that cardiacectomy was avoided in her operations. Most frequently in this work the corpora cardiaca were removed with the corpus allatum. After allatectomy alone, however, the effects were indistinguishable from complete extirpation of the ring gland. About 80 per cent of the flies operated upon in this way live apparently normally and show no external signs of their operation. Mortality is about 10 per cent and the remaining 10 per cent show the water balance upset described below.

Among the 80 per cent of operated flies that survived, normal mating reactions have been noted. The majority of flies (37 Lucilia, nine Sarcophaga) have been operated upon when 24 hours old, and subsequently fed sugar and water, and fixed after seven days. Thirteen cases in Lucilia were allowed to live to 21 days after the operation and three cases in the Sarcophaga series were fed meat for four days. However, eggs were never developed, though in one case a little yolk was found in an oöcyte slightly enlarged beyond stage I.

In spite of their normal behavior, operated flies present an unmistakable histological picture differing from the controls in regard to the fat body, ovary,

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PLATE II

Fat body of *Lucilia sericata* and *Sarcophaga securifera*. Fixed with alcoholic Bouin's. Photomicrographs, magnification 400 diameters, except Figure 7 which is ×170.

7. Fat body of normal male *S. securifera* three days of age. Note the larval fatbody cell whose fuchsinophil droplets have almost disappeared. Compare the oenocytes with those of the female (Figure 6). Note that the magnification is only 170 diameters.

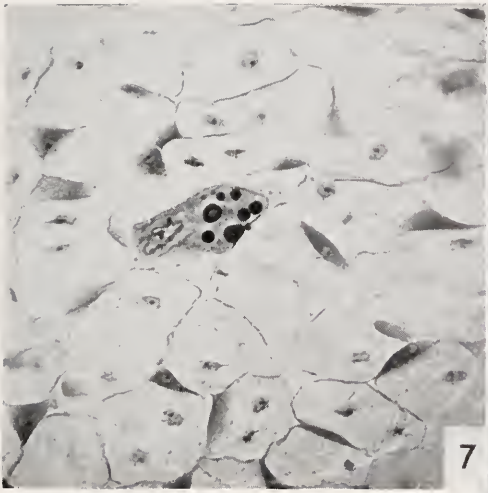
8. Fat body of female *L. sericata* in which recurrent nerve had been cut seven days. The reduced and pycnotic oenocytes, pycnotic small dark nuclei of the fat body are characteristic of flies after extirpation of the ring gland.

9. Fat body of male *S. securifera* allatectomized seven days. Note same effects as seen in Figure 8. The separation of the cytoplasm from the cell wall is characteristic.

10. Fat body of female *S. securifera* allatectomized for seven days when female was six days old. Note less marked effect than in Figure 9, though the effects of the operation can be seen in comparison with Figure 12.

11. Fat body of female *S. securifera* seven days after the extirpation of the ring gland with ring gland from female castrated seven days implanted for 46 hours. In comparison with Figure 9 the cytoplasm and nuclei are seen to have undergone conspicuous changes. Oenocytes, however, are unaffected.

12. As Figure 11, except that the transplanted ring gland was from a normal seven-day old female. Note that the fat body is almost normal, but the oenocytes still show the effects of allatectomy.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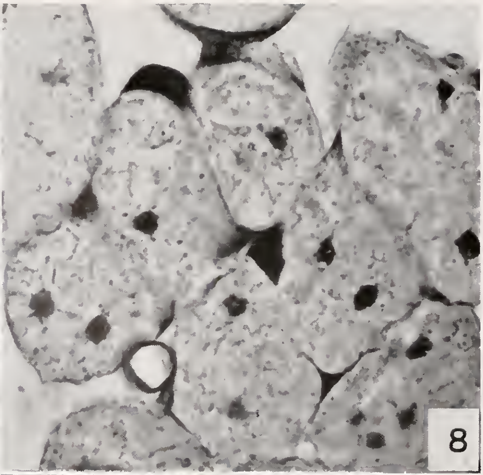
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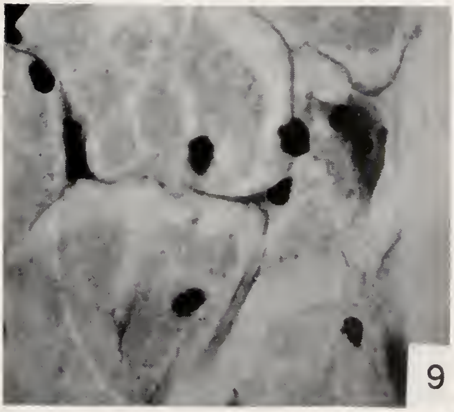
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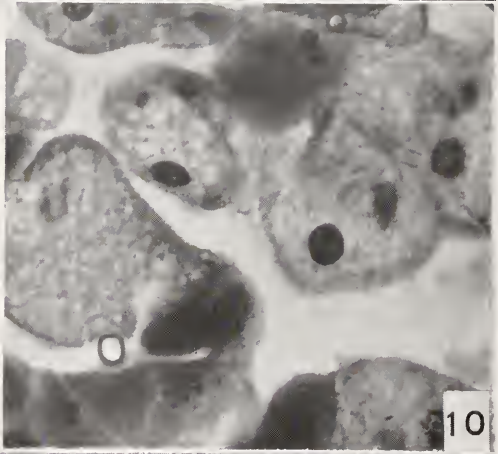
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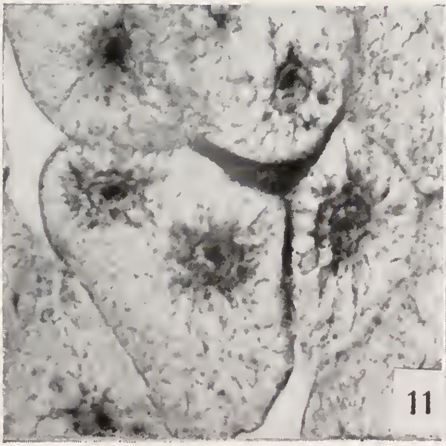
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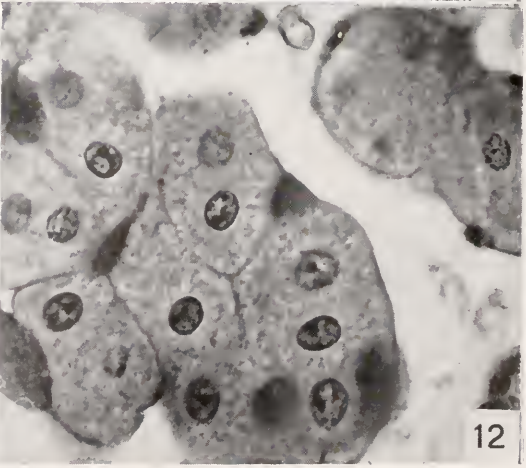
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PLATE II

and oenocytes. No effects could be seen on testes, on the nervous tissue, including the neurosecretory cells of the brain (Day, 1940), alimentary canal, Malpighian tubules, or on the accessory glands of the reproductive system of either the male or the female.

A. The effects on the fat body become visible within three days after the operation, but little change occurs from then up to 21 days. If the operation is performed on a fly within two days after emergence, the cells of the larval fat body never completely disappear, as they do in the normal fly. Even after the acidophilic cytoplasmic inclusions (see Figure 7) have disappeared, the larval cells can easily be recognized by the large size of the nuclei. Thus one of the effects of extirpation of the ring gland is to inhibit the normal maturation of certain tissues. The extent of the change in the adult fat body may be seen by comparing Figures 7 and 9. Many of the nuclei of the fat body cells in the operated flies become pycnotic, have crenulated borders, and are greatly decreased in size. A still more striking change occurs in the cytoplasm which appears much more sparse than in the controls. No specific stains have been used to attempt to learn exactly what components have disappeared from the cytoplasm but the general appearance is that of a cell whose reserves have been in large part utilized. It would be desirable to determine in what way these changes correlate with alterations in physiological function. The results suggest a comparison with the findings of Pfeiffer (1941) on Melanoplus that allatectomy results in a greatly increased fat content. While it will be seen from Figures 7 and 9 that there is an increase in fat body cell size following extirpation of the ring gland, an increase in fat does not appear to occur in female Lucilia or Sarcophaga after this operation although the vacuoles observed in the fat body cells may be left by dissolved fat.

If the operation is performed on a fly six days of age, the fat body does not regress, but the effect is still seen upon the oenocytes (compare Figure 10).

B. Even more striking changes occur in the oenocytes following extirpation of the ring gland. Such changes have not been previously reported for any insect. The effects are not quite comparable in Lucilia and in Sarcophaga. In males of the former species the large oenocytes of the male are most markedly affected, being much reduced in size. Their nuclei become pycnotic and their cytoplasm changes from an homogeneous basophilic to a strong acidophilic reaction. Comparable but less marked changes occur in the female Lucilia. In Sarcophaga the cytoplasm does not become so markedly reduced, but the cell boundaries of the oenocytes almost invariably become indistinct (Figure 9). As in Lucilia the nuclei show varying degrees of pycnosis and in both sexes the cells are greatly reduced in size. Feeding protein in addition to carbohydrate has little effect on these oenocytes.

C. Extirpation of the ring gland produces an effect upon the ovaries. If the operation is performed on young females fed only sugar, development beyond Stage 1 is never found. The histology of the eggs usually remains normal. However, in one case in which a female Sarcophaga was allatectomized when two days old, considerable regression of the ovaries was found. Practically no oöcytes were present and the ovaries consisted of a mass of knotted tracheae and a few small connective tissue and muscle cells.

If the operation is performed on a female in which the ovaries are well developed, degeneration of certain oöcytes occurs. Wigglesworth (1936) reported a similar situation in Rhodnius. The eggs on the periphery are affected first and the changes are exactly comparable to those seen in old females inhibited from ovipositing. Apparently extirpation of the ring gland hastens this process and causes its occurrence in a greater number of oöcytes than normal. The first indication of such degenerative changes is seen in an increase in size of the follicular cells. These large cells then begin to phagocytize the acidophilic yolk granules, and persist for a while with darkly staining masses in their cytoplasm. Eventually they digest this material and the yolk decreases in quantity until in the last stages that have been observed, the oöcyte contains practically no yolk.

Previous authors have described changes in the secretions of the oviduct as a result of allatectomy. These were not observed in Lucilia or Sarcophaga. Similar changes in the fat body and oenocytes occur in males as well as females, but the testes and accessory glands are unaffected.

D. As mentioned above, the effects of removing the corpus allatum just described are sometimes modified. In about 10 per cent of the operations the flies exhibit a marked distension of the abdomen, sometimes within five hours, but more usually about 12 hours after the operation. Complete serial sections of these cases shows no difference in the operation between these and the 80 per cent of flies which do not show these unusual effects. The Malpighian tubules are always considerably swollen and there appears to be an upset in the water regulating mechanism. This effect is found only in those flies which imbibe water and it is suggested that the operation may have stimulated some center, with the result that the flies imbibe more than they normally do.

THE EFFECT OF SEVERING THE RECURRENT NERVE

The question arises of whether the normal functioning of the ring gland is dependent upon its innervation. As shown by Day (1942) in Lucilia the recurrent nerve which supplies the ring gland is composed of fibers of three separate nerves. This compound nerve can easily be severed in the cervical region. This operation results in higher mortality than after extirpation of the ring gland. In one experiment, for example, 40 flies of both sexes of Sarcophaga were operated upon in this way, and of these only 16 survived for a period of one week. The effects of the operation are precisely similar to those following extirpation of the ring gland. The fat body cells enlarge, their nuclei become small and pycnotic, the oenocytes decrease in size and their cytoplasm becomes acidophilic, and the ovaries do not develop beyond Stage 1. The effect in Lucilia is shown in Figure 8 and that in Sarcophaga in Figure 10.

The histology of the corpus allatum after the severing of its nerve connections shows changes from the normal (Figure 3). The nerve endings are still conspicuous and little can be seen in the cytoplasm to suggest increased or decreased hormone output. However, striking changes occur in the cell size and in the nuclear size (Figure 3). Nuclear size is more easily measured than cell size. The nuclei of the operated flies measure from 12 to 14 microns in diameter, an increase of about 70 per cent over the diameter of the nuclei of the controls. The nucleolus also increases in size.

THE EFFECTS OF TRANSPLANTING RING GLANDS

Since it appeared from the extirpation experiments that corpora allata were concerned in maturation of the ovaries and directly or indirectly in the changes undergone by the fat body, the ring glands of ten-day females were transplanted into the abdomens of three-day females. Striking changes were induced in the hosts, probably attributable to the transplanted glands. The adult fat body of the host was markedly depleted, but the larval fat body cells still present showed a most unusual appearance as though their reserves were being mobilized more suddenly than is normal. This effect was found in individuals fixed 48 hours after the implantation. In flies fixed one week after the operation no larval, and extremely little adult fat body tissue could be found in sections. These experiments confirm the suggestion that the corpus allatum is concerned in the maturation of certain tissues.

A more significantly experiment seemed to be the implantation of ring glands into flies from which the ring gland had been extirpated for one week. It is hardly to be expected that the effects of extirpation of the ring gland could be reversed to the normal condition, for it has been shown that the normal activity of the corpus allatum is exhibited only when the gland is normally innervated. Two cases were fixed 46 hours after implanting the new gland. One showed an abundance of fat body, apparently intermediate between that of the fly from which the ring gland had been extirpated, and a normal of this age, and reduced oenocytes, with strongly basophilic cytoplasm rather than acidophilic as in flies after extirpation of the ring gland. The other showed a fat body and oenocytes which were essentially normal (Figure 12).

In a later section it will be shown that castration causes cytological changes in the corpus allatum of Lucilia, though not of Sarcophaga. However, the following experiment suggests that the corpora allata of castrate female Sarcophaga are physiologically altered. Ring glands of flies castrated seven days previously were transplanted as in the experiments just reported. Two cases were fixed 46 hours after implanting and two after seven days. Significant differences could be observed between these and the former series, but there are still definite effects of the implanted glands (Figure 11). These effects are sufficiently striking to confirm the suggestion that the ring gland from the castrated female had very different effects on the host from that from a normal female. It was noticed that the corpora allata of these transplanted glands, when studied in serial sections at autopsy, showed a slight indication of hypertrophy in a manner similar to that discussed above in denervated corpora allata *in situ*. Detailed analysis must await further experiments, but the generalization is warranted that castration causes physiological changes in the corpus allatum of Sarcophaga.

The Effects of Castration on Corpora Allata and Other Tissues

A. The experiments of Thomsen (1940) in which she extirpated ovaries were performed primarily on Calliphora. The operation resulted in hypertrophy of the corpora allata. Full confirmation has been obtained in my experiments with female *Lucilia sericata*, in which corpora allata showed considerable hypertrophy. The increase in size of the cells is illustrated in Figure 2 when compared with Figure 1. It will be noted that the increase is solely in cell size and there is no increase in cell number. The cytoplasm exhibits no more signs of activity than in the unoperated animals. Thomsen could offer no suggestion of the means by which the hypertrophy was brought about. A comparison with cases of hypertrophy of these glands, for example in Ephestia moths (Schrader, 1938) or termite royalties (Pflugfelder, 1938), does not assist, and it seems likely that a different mechanism is involved in each of these examples. There is a change in nuclear size comparable to that found in Sarcophaga, which results from severing the recurrent nerve.

B. Thomsen did not report experiments with male flies. These can be castrated even more easily than can females, for the testes are not so completely tracheated as the ovaries. However, no hypertrophy of the cells of the corpora allata, or any other change could be observed in male Lucilia either in behavior or in histology. Most cases were fixed seven days after the operation, but in a few cases even after 14 days no change could be observed.

C. Similar experiments were performed on Sarcophaga. Early observations indicated no hypertrophy of corpora allata. More extensive and detailed operations on both sexes were performed and the results carefully checked in histological preparations to determine whether castration was complete. No hypertrophy of the corpora allata comparable to that in female Lucilia was found in either sex of Sarcophaga (Figures 4 and 5).

D. It was thought that the accessory glands might have an effect. In male Sarcophaga the accessory glands alone and the accessory glands together with the testes were successfully removed. No change in corpora allata cells was found in ten operations.

In an activity apparently so fundamental in the physiology of the insect it is surprising that two genera as closely related as Lucilia and Sarcophaga should give such divergent results. Further discussion of their differences will be found on p. 139.

Castration of female Sarcophaga has no visible effects on the female accessory glands, or indeed on the majority of tissues. About 50 per cent of nuclei of fat body cells do, however, show varying degrees of pycnosis. The cytoplasm appears normal and the oenocytes are fully rounded and typical for this fly (Figure 6). No histological evidence was found in Sarcophaga to compare with the decrease in fat reported for castrated Melanoplus by Pfeiffer (1941).

The Effects of Cutting and Replacing Ovaries

In Sarcophaga an attempt was made to gain some indication of the effects on the ovaries by completely removing them from their attachments, and replacing them in the haemocoele. The flies were fixed after a period of one week and studied histologically. The ovaries had regained new tracheal connections, and appeared normal in every respect, with stage I oöcytes, as would be expected in flies fed only sugar. Fat body and oenocytes were normal and no effect of the operation was observed on the corpora allata.

General Discussion

We may assume for purposes of comparison with other insects that the effects of extirpation of the ring gland can be compared with allatectomy and cardiacectomy. The only report of cardiacectomy is to be found in a note by Pfeiffer (1939, p. 452–453) stating that "delay in molting has been consistently obtained by removing the corpora cardiaca" of Melanoplus. Allatectomy has been performed by Wigglesworth (1936) on Rhodnius where it was shown to result in loss of the ability to produce mature eggs. Degeneration occurred not only in the oöcytes but also in the follicular epithelium. Weed (1936) confirmed these results with Melanoplus. However, in Dixippus (Pflugfelder, 1937) allatectomy does not result in the loss of the ability to produce mature eggs though the fact that the corpora allata have some effect on the ovaries is shown by subsequent work (Pflugfelder, 1940). Subsequently Pflugfelder has reported a variety of effects from the removal of the corpora allata of Dixippus: the pericardial glands and ventral glands undergo considerable hypertrophy, and there are effects on the regeneration of lost limbs (1938b). These results lead to the conclusion that the corpora allata exert some influence on metabolism, but as Scharrer (1941) says "the question is how far this concept may explain all the special effects attributable to the glands."

From the present experiments there is evidence that the ring gland produces more than a single substance. As has been pointed out they indicate strongly that one of the primary effects of the ring gland is on the regulation of normal maturation. This is also true in the larva, as shown by the experiments of Burtt (1938) on Calliphora and of Hadorn and Neel (1938) on lgl Drosophila larva. Histological examination of "permanent larvae" of *Lucilia sericata* inhibited from pupating by removal of the ring gland shows a fat body which is unlike anything seen in normal larvae. The cells contain a large number of small acidophilic droplets, and a few larger droplets which stain with aniline blue. However, there is no regression or deterioration of the fat body cells as found in the adult fly, and a somewhat comparable picture is seen in a prepupa kept for one month in dry sand (see Mellanby, 1938). In such fat body cells many of the acidophilic droplets are considerably larger, are less regular in size, and are aggregated around the nucleus.

It is not known whether the hormone which permits normal pupation and whose removal results in fat body cells of this type is the same as that which plays a role in the removal of larval fat body in the adult fly. It seems unlikely that this is the case. And there is sufficient change in the ring gland that it is not necessary to assume that a single hormone is involved. A few experiments have been performed of transplanting glands from larvae to adults. Though they yielded no significant information, the conclusion that the ring gland produces a substance concerned with normal maturation seems incontravertible.

It has been shown that the effects produced by implanting a ring gland into a fly from which the ring gland had been extirpated are qualitatively different from those which result from severing the innervation of a gland *in situ*. It is possible that the effects may be produced by different concentrations of a single secretion but it appears likely that the substance causing the breakdown of larval tissues is not the same as that which affects the growth of the ovaries, the cytoplasm of the fat body, and the structure of the oenocytes (see also Vogt, 1940). As has been suggested by Pflugfelder the corpus allatum seems to have some influence on the metabolism of the insect. The obvious effects of the corpora allata of various insects on the ovaries have led to the suggestion that they may produce a hormone acting directly on the sex organs, at least in the female. The outstanding result of the experiments reported in this paper is that many tissues are affected by the corpus allatum. There is thus no reason to suppose that a sex hormone is produced by the corpus allatum. In fact, it seems more plausible to assume that the primary effect is on some general metabolic function. It is well known that many flies are unable to mature their eggs without a protein meal, while mature sperm are formed irrespective of the meal obtained by the male. Spermatogenesis is in no way affected by extirpation of the ring gland, while eggs, if formed, begin to undergo regression if the ring gland is removed even though protein be fed to the flies.

The results of castration are difficult to interpret. In female Lucilia, the only cells markedly affected are those of the corpora allata. In male Lucilia and in both sexes of Sarcophaga, castration has little effect either on the behaviour of the flies or on the cytological appearance of their tissues (see Figure 6). In the female Lucilia the corpus allatum hypertrophies after castration, and in this fly the oenocytes of the female are much smaller than those of the male. In Sarcophaga, castration does not result in any cytologically visible change in the corpus allatum of either sex, and in this fly the oenocytes of the female are almost indistinguishable from those of the male. This suggested that changes in the oenocytes might be induced by castration of female Lucilia and of male Sarcophaga, but none were found. However, this finding does not invalidate the general conclusion that the effect of the ring gland is probably primarily on some general metabolic process, perhaps acting through the oenocytes rather than directly on the ovary.

SUMMARY

1. Evidence from extirpation and transplantation experiments suggests that the ring gland of *Lucilia sericata* and *Sarcophaga securifera* produces a hormone concerned with normal development. Its action can be seen in the larva where it results in puparium formation, and in the adult fly first in the changes which occur during the breakdown of the larval fat body cells and subsequently in the changes undergone by the adult fat body cells, the oenocytes, and the development of the ovaries.

2. These last two activities may be under the influence of a hormone (probably different from that influencing development), whose action seems to be on the general metabolic activity of the fly. The oenocytes undergo marked changes after extirpation of the ring gland. If these are concerned with some general metabolic function, as seems likely, the action may be primarily on them and the effects on fat body cells may be altered by implanting a ring gland into the abdomen of a fly, after extirpation of the ring gland, but this has no visible effect on oenocytes or on ovarian development.

3. Castration of adult female *Lucilia sericata* results in hypertrophy of the cells of the corpus allatum. No effect is produced in the male *Lucilia sericata* or in either sex in *Sarcophaga securifera*.

4. Destruction of the innervation of the ring gland of *Sarcophaga securifera* results in slight hypertrophy of the corpus allatum cells, and of their nuclei. The physiological significance of this hypertrophy is not yet known.

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CHANGES IN VOLUME AND PHYSICAL PROPERTIES OF ALLANTOIC AND AMNIOTIC FLUIDS UNDER NORMAL AND EXTREME TEMPERATURES

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Avian embryonic membranes develop as special temporary organs. They begin to appear early, developing quite separately from those of the embryo, and cease to function at hatching. Their presence is indispensable because they provide both for the protection of the embryo and for its independent existence. They participate in nearly all metabolic activities of the embryo, such as nutrition, respiration and excretion.

In spite of the biological importance of these membranes, little is known about their development. A study of the changes in volume and physical properties of the allantoic and amniotic fluids under both normal and extreme temperatures would be of especial value in a better understanding of the physiology of development of the avian embryo. Therefore, with these ideas in mind the present work was undertaken.

Experimental Methods

About 650 fertile eggs of White Leghorn hens (*Gallus domesticus*), 200 of Ringnecked pheasants (*Phasianus torquatus*), 150 of Bobwhite quail (*Colinus virginianus*), 200 of White Holland turkeys (*Meleagris gallopavo*) and 200 of Pekin ducks (*Anas domesticus*) were used. The eggs were incubated in the laboratory incubators previously described (Romanoff, 1932). Normal conditions for development were a temperature of 37.5° C., relative humidity of about 60 per cent, and an air movement of about ten feet per second, only with some minor modifications according to the specific requirements of each species. There was a sufficient supply of fresh air and an adequate removal of carbon dioxide. All of the eggs were turned at regular intervals three times a day.

For a more detailed study of the effect of temperature on the development of embryonic membranes—allantois and amnion—chicken eggs were selected. Eggs of other species were not used because of their scarcity.

The experimental temperatures for incubation of the chicken eggs were 34.5°, 36.0°, 38.5°, and 39.5° C. All of the other environmental factors were similar to those under normal conditions.

The volumes of allantoic and amniotic fluids were measured with the aid of a special aspirator. After removing the shell and shell membranes at the blunt end of the egg, the allantoic membrane is pierced by the large hypodermic needle on the aspirator. The allantoic fluid is withdrawn into the small measuring cylinder by pressing the suction bulb until the total volume of liquid is obtained. The amniotic fluid is withdrawn in a similar manner, except that the older embryos may be placed in a clean Petri dish to facilitate removal of the liquid.

After the amniotic membrane is pierced, any escaping liquid can be collected directly from the dish. On the whole, this method permits quick and accurate measurements of the fluids and their ready use for further immediate studies.

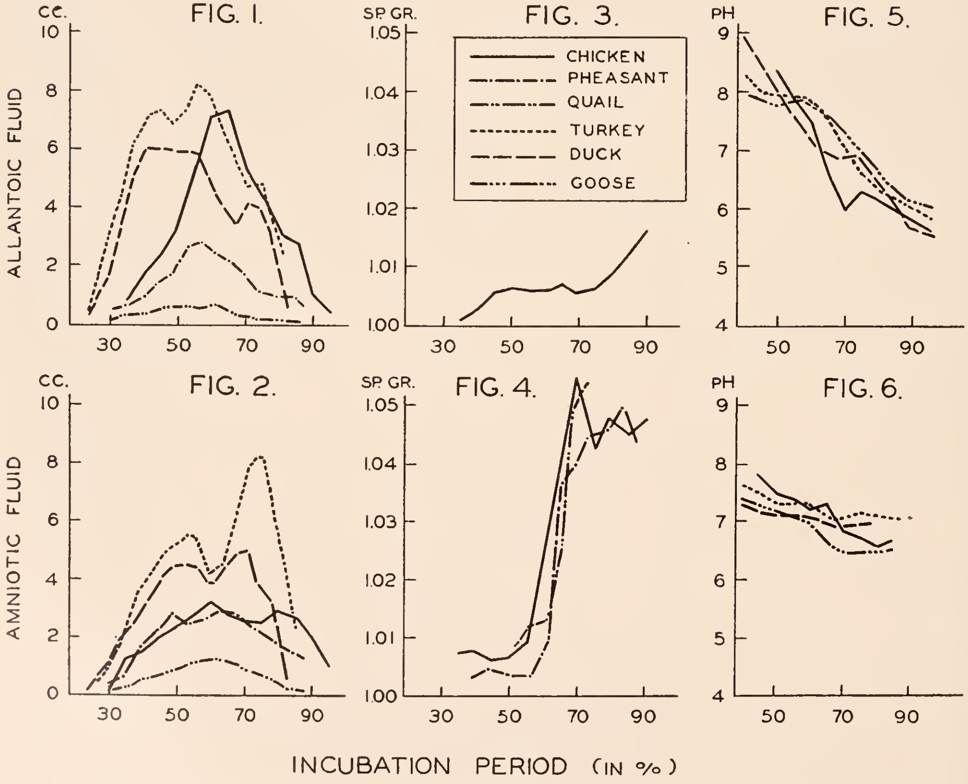
The specific gravity of the fluids was determined by weighing them in small standardized pycnometers of approximately 0.5 cc. capacity. These pycnometers were made in 14 mm. lengths from glass tubing with a 3 mm. bore. In a test determination by this method the average deviation of density was about 0.00024.

The hydrogen-ion concentration (pH) of the fluids was measured electrometrically, using a hydrogen electrode of special design for small quantities of material of about 1.0 cc.

EXPERIMENTAL RESULTS

Volume of fluids under normal conditions

The actual volume of allantoic and amniotic fluids in the eggs of the various species showed enormous variation (Figures 1 and 2), since the weight of the eggs used varied from about 9 grams for quail to about 85 grams for turkey.



FIGURES 1 TO 6. Changes in volume, specific gravity and hydrogen-ion concentration (pH) of allantoic and amniotic fluids of avian eggs incubated under normal conditions. (Abscissae represent the per cent of time for the full embryonic developmental period—chicken 20 days, pheasant and quail 23 days, turkey and duck 27 days, and goose 30 days.)

However, when the amounts are considered in relation to the size of the egg, it is found that the allantoic fluid at the peak of accumulation is approximately 9 to 10 per cent of the original egg weight, while the amount of amniotic fluid at its peak is about 8 to 9 per cent.

The peak for the allantoic fluid is reached shortly after the middle of the incubation period, and corresponds in general with the observations of Kamei (1927), and Ogorodniy and Penionschkevitsch (1939) on chicken eggs. The volume of fluid shows a fairly regular rise, and, after reaching the peak, it falls with nearly the same rate.

The duration of the period in which there is a high percentage of fluid in the egg is apparently longer with the amniotic than with the allantoic fluid. The peak of volume of amniotic fluid occurs somewhat later during incubation, and there is a slight depression in the curve occurring at approximately the time of highest allantoic accumulation. This depression was found to be greatly exaggerated with chicken eggs under natural incubation (Ogorodniy and Penionschkevitsch, 1939).

Specific gravity of fluids under normal conditions

The specific gravity of allantoic fluid rises throughout the period of incubation, increasing most rapidly during the early and late stages of development (Figure 3). On the other hand the specific gravity of amniotic fluid (Figure 4) reaches a peak at about two-thirds of the incubation period and rises most sharply during the time in which the allantoic fluid shows the least change. The data agree with a few observations of Kamei (1927), and Ogorodniy and Penionschkevitsch (1939) for chicken eggs.

Hydrogen-ion concentration of fluids under normal conditions

With both allantoic and amniotic fluids of chicken eggs, as well as of turkey, duck and goose (*Anser domesticus*) eggs (Shklyer, 1937), there was found to be a definite trend in hydrogen-ion concentration (pH) during the period of incubation (Figures 5 and 6). In the allantoic fluid it decreased at a moderate rate from high alkalinity at about the mid-period to medium acidity at the time of hatching. On the other hand, it showed a more direct relationship with the amniotic fluid and with the stages of incubation. In general, it dropped steadily from medium alkalinity to slight acidity. The hydrogen-ion concentration of the amniotic fluid decreased at a slower rate than that of the allantoic fluid and thus was limited to a narrower range.

In some data presented by Aggazzotti (1913), Gueylard and Portier (1925) the hydrogen-ion concentration for allantoic fluid seemed to follow a curve convex to the abscissa but always near neutrality.

Volume of fluids under extreme temperatures

The accumulation of allantoic fluid of chicken eggs was noticeably influenced by abnormal temperatures (Figure 7). The greatest volume of 7.3 cc. occurred under normal temperature, while the next highest level of 6.7 cc. occurred under slightly abnormal temperatures of 38.5° and 36° C. With increasingly abnormal conditions at 39.5° and 34.5° C. the volume level was reduced to approximately

4.5 cc. Earlier work (Romanoff, Smith and Sullivan, 1938) showed a still lower level of the fluid, less than 2 cc. at 40.5° C.

Concomitantly there occurred a shifting of peaks of accumulation, the peak occurring early under high temperature and late under low temperature. This coincides with the growth response of the embryo to abnormal temperatures (Romanoff, Smith and Sullivan, 1938).

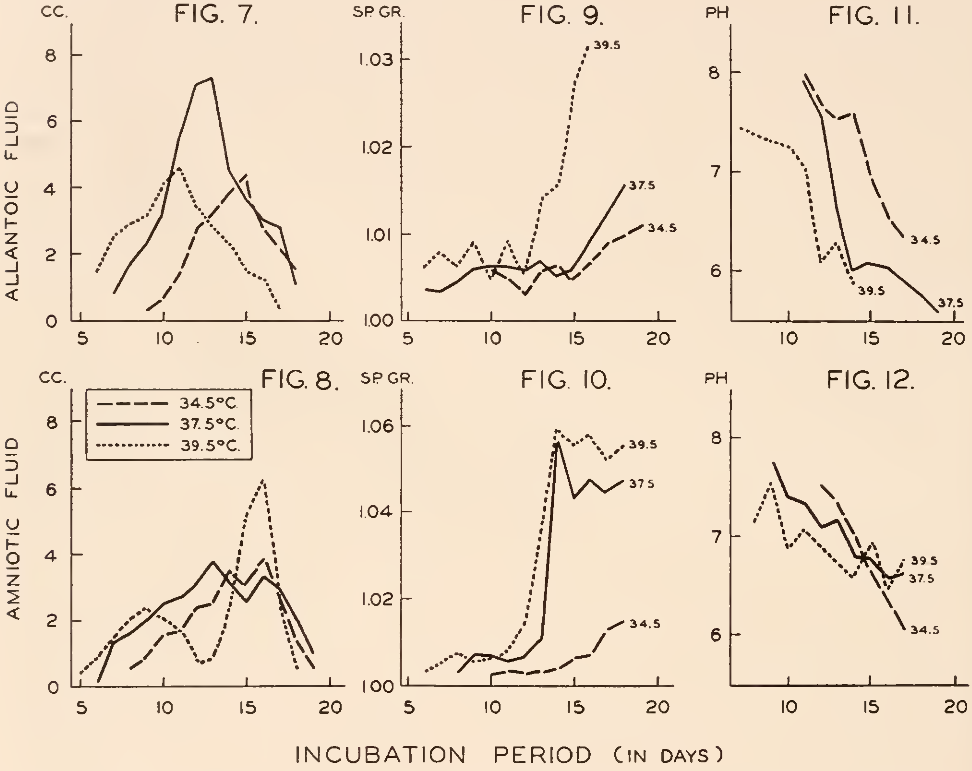


FIG. 7. FIG. 9. FIG. 11.

FIG. 8. FIG. 10. FIG. 12.

FIGURES 7 TO 12. Influence of incubating temperature on the volume (Figures 7 and 8), specific gravity (Figures 9 and 10) and hydrogen-ion concentration (pH) (Figures 11 and 12) of allantoic and amniotic fluids of chicken eggs.

Normally there were observed two successive almost equal peaks of accumulation of amniotic fluid (Figure 8). The time of occurrence of the first peak was noticeably affected by the temperature; it occurred early under high temperature and late under low temperature. This also seems to follow the general growth response of the embryo to various temperatures.

The depression occurring between the peaks of volume is wider at high and narrower at low temperature since the second peak occurs at approximately normal time. This second point of maximum volume is extremely exaggerated under both high temperatures 38.5° and 39.5° C., and follows a much greater depression than occurs under lower temperatures. Similar changes in volume of amniotic fluid were observed by Ogorodniy and Penionschkevitsch (1939) at incubating temperatures of about 39° C.

Specific gravity of fluids under extreme temperatures

The physicochemical properties of the fluids of chicken eggs were also definitely altered. There was a marked tendency for the specific gravity of the allantoic fluid to increase at a faster rate with higher temperature (Figure 9). Furthermore, this increase occurred earlier during incubation with higher temperatures, although all values increased, in general, with the corresponding morphological age of the embryo.

The amniotic fluid at the beginning showed a slightly lower specific gravity than the allantoic fluid under similar conditions (Figure 10). Then at the mid-period of the development with high temperature it rose very rapidly and remained at a somewhat higher level than under normal conditions. With low temperature the rise in specific gravity was delayed and on a whole insignificant. This gave great differences in value under the extreme temperatures.

Hydrogen-ion concentration of fluids under extreme temperatures

The allantoic fluid showed a delayed initial decrease in hydrogen-ion concentration with a decrease in temperature (Figure 11), but the rate of decrease in values under all conditions was at approximately the same rate.

The change in hydrogen-ion concentration of the amniotic fluid (Figure 12) was almost lineal with development, the values decreasing more rapidly at low and more gradually at high temperatures.

Discussion

The changes in volume and composition of both allantoic and amniotic fluids are closely associated with the water metabolism of the avian egg. From the fact that there is a plentiful supply of water in the egg, Gray (1926) suggested that this makes it possible for the eggs of land vertebrates to develop without an external water supply such as that provided for their fish-like ancestors. The formation of an amnion and an allantois in an avian egg is therefore possible, with the aid of osmotic and other physicochemical activities within the egg, in spite of a continuous loss of water by evaporation. According to Needham (1931), the osmotic pressure of the embryonic body, for example, rises steadily as development goes on, that of the amniotic liquid stands more or less stationary, and that of the allantoic liquid greatly declines. This in turn may have an intimate relationship to the specific gravity of allantoic and amniotic fluids.

The increase in volume of allantoic fluid is required to assist in the excretion of uric acid (Fiske and Boyden, 1926; Romanoff, Smith and Sullivan, 1938). The reabsorption of water from the allantois must begin very soon after the mid-period of development, because its uric acid content is increasing, while its volume is remaining steady or diminishing (Romanoff, Smith and Sullivan, 1938). The uric acid maximum follows ammonia and urea maxima during ontogenesis (Needham, 1926). In fact, the change in hydrogen-ion concentration of the allantoic fluid during the last half of incubation from an alkaline to an acid state has been explained in the relationship of urea and ammonia to uric acid (Aggazzotti, 1913). It has been shown (Fiske and Boyden, 1926) that the allantois is functional in the excretion of nitrogeneous waste products as early as 2.5 days.

The excessive secretion of allantoic fluid then apparently coincides with the maximum for uric acid in order to assist in the excretion of the nitrogenous waste.

On the other hand, the amniotic fluid reaches what is practically its maximum by the mid-period of incubation. It was found by Ogorodniy and Penionschkevitsch (1939) that penetration of large amounts of albumen into the amniotic cavity is responsible for this increase in volume, and also for the increase in its protein content. These lead to a greatly heightened viscosity of amniotic fluid. Close to the end of the developmental period, however, the embryo swallows up a large quantity of amniotic fluid and its volume rapidly decreases.

The marked reduction in volume of allantoic fluid at both high and low temperatures would indicate the underdevelopment of the allantois. This presumably is associated with the developmental restrictions in extra embryonic circulation. The observations of Tazelaar (1928) and Romanoff (unpublished) show that during the first week of incubation there is a noticeable maldevelopment of the area vasculosa. Also the presence of greater amounts of uric acid during later stages (Romanoff, Smith and Sullivan, 1938) gives further indications that the metabolic equilibrium of the embryo is upset by adverse thermal conditions.

The changes in physical properties of both fluids, as was anticipated, were largely in line with the accelerated or retarded development both of the embryo and of its membranes as induced by high or low temperatures.

SUMMARY

Under normal conditions of incubation the relative volume and physical properties of allantoic and amniotic fluids are nearly identical in chicken, pheasant, quail, turkey, duck and goose eggs.

Under high and low temperature the fluids are altered in respect to both volume and physical properties. In general, the course of changes follows the morphological age of the embryo. The volume of allantoic fluid was suppressed, while that of amniotic fluid was excessively enlarged during the later part of incubation under high temperature. The time of increase in density was shifted in both fluids along with the developmental stage of the embryo; there was also a noticeable reduction in the density of the amniotic fluid at low temperature. The drop in hydrogen-ion concentration of allantoic fluid was nearly identical, except for the time factor, while the values for amniotic fluid decreased more rapidly at low and more gradually at high temperatures.

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PHYSIOLOGICAL OBSERVATIONS UPON A LARVAL EUSTRONGYLIDES. IV. INFLUENCE OF TEMPERATURE, pH AND INORGANIC IONS UPON THE OXYGEN CONSUMPTION

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Experiments described in two previous papers of this series (von Brand, 1938, 1942) showed conclusively that a larval Eustrongylides, occurring encysted in Fundulus, leads a predominantly oxidative life while in the fish. The evidence was derived on the one hand from experiments comparing the glycogen consumption of aerobically and anaerobically kept Eustrongylides larvae with that of animals living under natural conditions either purely oxidatively (earthworm, etc.) or primarily anoxidatively (Ascaris). The second approach consisted in comparing the gaseous exchange of freshly isolated worms, with that of larvae previously exposed to a period of anaerobiosis. The fact that this larval nematode has an oxidative metabolism is of considerable interest, since with the exception of Trichinella larvae (Stannard, McCoy and Latchford, 1938), most parasitic worms that have so far been studied possess a predominantly fermentative type of metabolism. It seemed of interest therefore to investigate the influence of various factors upon the oxygen consumption of this worm.

Material and Methods

As in previous experiments only medium-sized to large worms were used, and, depending upon the size, from four to five of these larvae constituted an experimental lot. The oxygen consumption was again determined by the Warburg method. The details of the experimental procedure have been described adequately in the second paper of this series (von Brand, 1942). It should be added that the vessels were shaken 100 times per minute with an amplitude of 3 cm. Control experiments showed that this rate was sufficient to establish a satisfactory equilibrium between air and water. The temperature and the saline solutions used varied in the different series, the details will be given at the appropriate places in the following sections.

Influence of Temperature

One factor that under natural conditions will be of the greatest importance in determining the metabolic level of Eustrongylides is the temperature. The worms in the intermediate host are, during the varying seasons of the year, exposed to temperatures ranging from near the freezing point to about 25° C. Cowles (1930) found during his survey of Chesapeake Bay in his area U near Baltimore, surface temperatures ranging from 0.3° C. to 24.8° C. and bottom

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[1] The author is indebted to the Elizabeth Thompson Science Fund for a grant towards the purchase of the respiration apparatus used in this investigation.

temperatures varying between 0.9° C. and 24.4° C. Once the parasites enter the definitive host, probably a heron, they are exposed to the higher, but constant temperatures characteristic of birds. An investigation of the temperature influence upon the oxygen consumption seemed therefore desirable and the more so, as aquatic animals—parasites must be compared with them rather than with terrestrial forms—encountering such a wide range of temperature fluctuations in their normal surroundings are relatively rare. Furthermore with the exception of a few experiments of McCoy (1930) on infective larvae of *Ancylostomum caninum* no work on the temperature relationships of the respiration of helminths seems to have been performed.

The results of the temperature experiments are summarized in Table I. For each experiment freshly isolated worms were used. This, of course, introduces the biological variation between various lots as a source of error. It seems, however, preferable to use this method rather than to employ the same worms for determinations at several or all temperatures. This would have required long periods outside the host, during which time the metabolism sinks somewhat, even under the most favorable conditions. It is believed that the number of experiments is sufficient to eliminate gross errors due to biological variation. Most experiments were conducted for four hours with readings at half hour intervals. Below 17° C., the readings were taken only hourly, and at 5° in two to two and one-half hour intervals. In these cases the experimental periods extended up to eight hours. All temperatures with the exception of that of 48° C. were well tolerated by the nematodes. At 48°, however, the O_2 consumption was high only for two hours. It then sank rapidly, indicating an injury to the worms. They in fact became more or less rigid and did not recover. At 45° C., on the other hand, the worms survived well; after the experiments they were kept for several days at room temperature and showed normal motility during this time.

TABLE I

Oxygen consumption of a larval Eustrongylides at different temperatures

Temperature ° C.	Determination number	cmm. O_2 consumed/gm./half hour	
		Mean value of all readings	Mean value after exclusion of first reading
5	8	4.5 ± 0.4	3.5 ± 0.3
10	7	9.1 ± 0.6	7.3 ± 0.6
17	8	17.0 ± 1.7	15.7 ± 1.4
22	7	35 ± 4.1	32 ± 4.6
27	7	54 ± 5.7	53 ± 5.7
32	7	64 ± 10	62 ± 10
37	19	76 ± 4	75 ± 4
42	7	95 ± 8	90 ± 7
45	8	113 ± 5	107 ± 4
48	7	131 ± 13	117 ± 16

At all temperatures the first reading was somewhat higher than were the subsequent ones. This is due, as discussed previously (v. Brand, 1942), to the repayment of a small oxygen debt contracted in the cysts. The following discussion is based on the mean values obtained after excluding the first reading, but the conclusions reached would not be changed materially if the first values were included.

An analysis of the values obtained shows that the increase in O_2 consumption with rising temperatures does not follow Krogh's (1914) normal curve. Two curves were necessary to express the temperature relationships adequately. The Q_{10} of the temperature interval 5 to 27° C. is 3.55, whereas in the range of 27 to 48° C. a Q_{10} of 1.48 is found (Figure 1). Similarly two bisecting lines resulted if the values were calculated according to Arrhenius' formula (Figure 2). It is of interest to note that 27° C. is one of the critical temperatures where according to Crozier (1926) a break in the curves is frequently found. The μ values too are well within the range of those frequently found in biological processes (Crozier, 1926*a*). These findings might be interpreted with Crozier (1925) on the assumption that two master reactions are involved. One might even be tempted to correlate them with the life history of the worm: One of the master reactions would conceivably control the metabolism of the larva in the fish—the turning point of the curves is near the highest temperature to which Fundulus

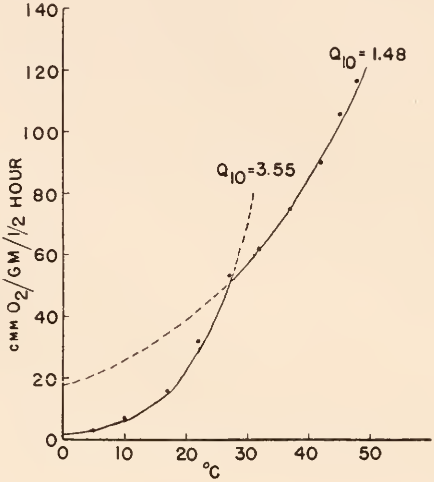

FIGURE 1. The Q_{10} of the oxygen consumption of a larval Eustrongylides.

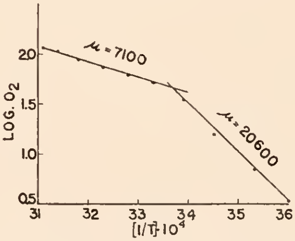

FIGURE 2. The oxygen consumption of a larval Eustrongylides expressed according to Arrhenius' formula.

is exposed in this region, the other would be characteristic for the processes proceeding once the definitive host is reached. I am, however, at the present time not prepared to draw such a sweeping conclusion: it should be remembered that several investigators (Bělehrádek, 1935; Ponder and Yeager, 1930) have raised serious objections against Crozier's interpretations of the significance of bisecting lines resulting from the application of Arrhenius' formula. Bělehrádek (1935) has proposed a formula that frequently allows the temperature relationships to be expressed by a straight line, where other formulae require two. The Eustrongylides values are presented, according to Bělehrádek's formula, in Figure 3. They show a fairly good fit to a straight line. It should be remembered, however, that the constant α is purely arbitrary, and has to be chosen differently from case to case. If it is changed even a few degrees the single line relationship does not hold in the present case. It seems to the writer that further progress in the question of temperature relationship might be expected from a simultaneous investigation of various metabolic processes at different temperatures.

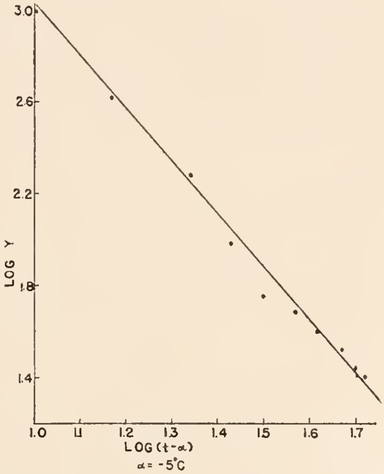

FIGURE 3. The oxygen consumption of a larval Eustrongylides expressed according to Bělehrádek's formula.

INFLUENCE OF pH

Another factor that may vary considerably in the natural environments of Eustrongylides is the hydrogen ion concentration. Although no actual data are available it can perhaps be expected that the cyst fluid formed by the fish may, like many other secretions of the body, have a pH fluctuating around the neutral point. But as soon as the worm is freed from the cyst in the body of the definitive host the situation becomes different. It is reported to live in the glands of the fore stomach of aquatic birds or in the fat around the gizzard, but it has also been recovered from the intestines and omentum (Jaegerskioeld, 1909; Cram, 1934). Hunter (1937) found immature adults threaded in and out the stomach wall of the black crowned night heron and the little green heron. Before establishing itself in its final position the worm will be exposed to the digestive juices. No data on the pH of these heron species were found in the literature, but Mennega (1938) investigated the pH of the stomach content and stomach wall of a European species of heron. She found after 24 hours starvation, pH values of 2.40 and 3.10 respectively for the fore stomach, while the pH of the contents varied from 3.56 to 6.10 and those of the wall from 4.14 to 5.75 after food had been taken in 45 minutes to $3\frac{1}{2}$ hours prior to the determinations. Cases in which the respiratory rate varies in solutions of different pH are known from organisms belonging to various phyla. In the holothurian Thyone, for example, Hiestand (1940) found a steady increase in respiration in the pH range 5.4 to 8.8, whereas Hiestand and Hale (1938) found an increased O_2 consumption in fresh water molluscs when the pH was lowered. Maier and Coggeshall (1941) found that the rate of O_2 consumption of a malaria parasite (*Plasmodium Knowlesi*) remained constant between pH 7.0 and 8.0, but declined rapidly between pH 8.0 and 9.0. Cook and Sharman (1930) found a marked influence of the pH on the CO_2 output of Moniezia, but their results need confirmation since the experimental periods used exceeded by far the length of time in which these worms remain normal in inorganic solutions according to the experience of other investigators.

The parasitic nematodes seem never to have been used for a study of this type. Since the pH seems to be at least in some cases a limiting factor in the distribution of these parasites in the host (Davey, 1938), a study of the influence of pH on the O_2 consumption was undertaken. In order to get and maintain the intended hydrogen ion concentrations, Sörensen's phosphate (1/15 molar) and citrate buffers (1/10 molar) were used. The necessary amounts of NaCl were added to make the solutions isotonic to a one per cent NaCl solution. Three to five worms were isolated from the fish and kept for 24 hours at 37° in about 30 cc. of the solution in order to adapt them as far as possible to these media. After 24 hours the O_2 consumption was determined over a period of four hours. For each experiment a new batch of two to five worms was used, the temperature was 37° C. A total of 74 experiments was performed, covering the pH range of 1.13 to 10.60. The worms withstood the total period of 28 hours in these solutions without harm, their viability was controlled by transferring them after the determinations to one per cent saline for two days during which time they invariably showed normal motility. The extreme acidities and the alkalinity of pH 10.6 could, however, not be tolerated for a much longer period. Separate experiments showed that the worms cannot be kept longer than about two to three days in these solutions at 37° C. without permanent injuries, whereas they lived considerably longer in the other solutions.

The results are given in Figure 4. It is apparent that between pH 3.4 and 8.3 the O_2 consumption remains on an average quite constant, the average rate being 76 cmm./gm./$\frac{1}{2}$ hr. The only exception occurred at pH 4.5, where a somewhat higher O_2 consumption was found in a phosphate buffer solution. This solution contained only KH_2PO_4 + NaCl. It seems possible that the increased rate may be only an expression of the biological variability, but it is equally possible that it is due to a stimulating effect of the K ion, which is pronounced in pure solution as will be shown in a following paragraph. In the acid range a distinctly increased rate of O_2 consumption was found at pH 1.13 and 1.80. This, probably, as an observation of the worms shows, is due to an increased muscular activity. This may be of biological significance. The worms have to bury themselves into the mucosa of the fore stomach and are then probably exposed to a very low pH. This may then be the stimulus necessary for the initiation of the boring movements.

An increased O_2 consumption was also observed in the extreme alkaline range. This is probably without biological significance, since it seems unlikely that the nematodes will in their natural environments be exposed to similar conditions. It may be assumed that this is an expression of increased activity due to unfavorable surroundings.

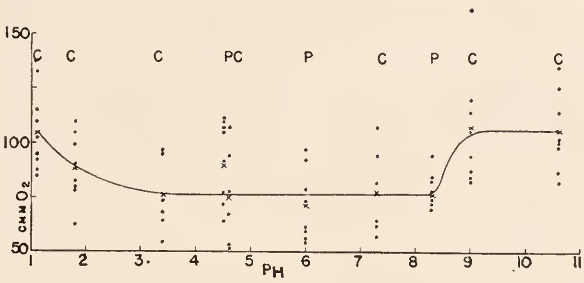

FIGURE 4. Oxygen consumption of a larval Eustrongylides in salines of various pH. Dots—single determinations, crosses—mean values. P—Phosphate buffer, C—Citrate buffer.

INFLUENCE OF INORGANIC IONS

Experiments discussed in a previous paper (von Brand, 1942) showed that the molecular concentration of the medium could be changed in rather wide limits without interfering with the oxygen uptake of the parasite under consideration, or without changing its life in vitro to a marked extent (von Brand and Simpson, 1942). These findings were believed to be of biological importance, since the complicated life cycle of this worm, although incompletely known, includes actually a variety of natural "media." The eggs are probably passed out from the host into water, possibly both fresh and brackish water, judging from the regions from which the parasites have been reported. It seems likely that two intermediate hosts are involved; the first is not yet known definitely, but is probably a crustacean. As second intermediate host a variety of different fish may serve, and, as already mentioned, aquatic birds are the definitive hosts. Besides differences in concentration, differences in chemical composition, both in regard to organic and inorganic constituents also may be expected in these various habitats. Since inorganic ions frequently have a marked influence upon respiration (some of the pertinent facts are reviewed in Heilbrunn, 1937, and Canzanelli, Rogers and Rapport, 1942), the respiration of Eustrongylides was studied in solutions of various ions.

Solutions isotonic to a 1.0 per cent NaCl solution were used throughout this work. The following cations were used in the form of their chlorides: Na, Mg, Ca, NH_4, K, and the following anions in the form of their sodium salts: Cl, SO_4, NO_2, NO_3 and PO_4. The pH of all solutions was in the range not affecting the oxygen uptake, in the case of the PO_4 ion this was achieved by using a mixture of equal parts of Na_2HPO_4 and NaH_2PO_4. All experiments were conducted at 37° C. and for each solution eight different lots of worms were used. Determinations of the oxygen consumption were performed immediately after the freshly isolated worms had been washed thoroughly with the respective solutions,

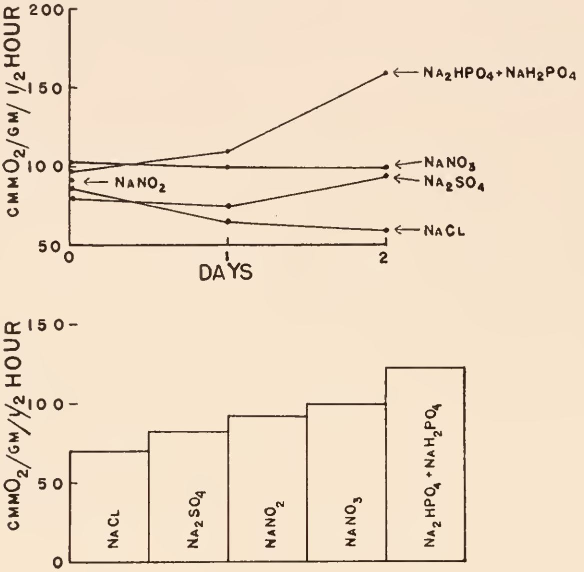

FIGURE 5. Oxygen consumption of a larval Eustrongylides in isotonic solutions of various anions. The upper part of the figure shows the average O_2 consumption on specified days, the lower part the average O_2 consumption over the entire period of observation.

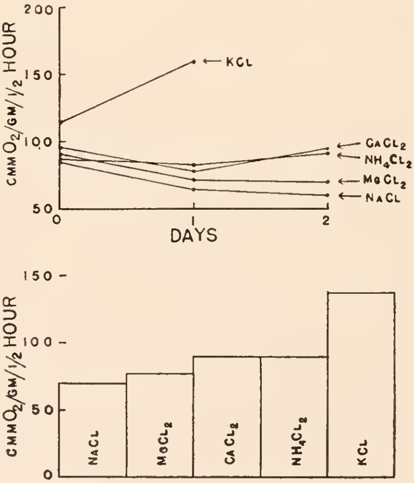

FIGURE 6. Oxygen consumption of a larval Eustrongylides in isotonic solutions of various cations. The upper part of the figure shows the average O_2 consumption on specified days, the lower part the average O_2 consumption over the entire period of observation.

after 24 hours and 48 hours. The parasites were kept in 30 cc. of the various solutions at 37° C. between the determinations. It was, however, impossible to secure the 48 hours value in the case of KCl or the 24 and 48 hours values in the case of $NaNO_2$, since the worms did not survive long enough. All the other solutions were tolerated remarkably well. It does not seem very likely that the well known relative impermeability of the nematode cuticle is the responsible factor since the total average survival was longest in NaCl and definitely shorter in all other solutions. An exception is perhaps $MgCl_2$. The worms kept in this solution for days showed no sign of anesthesia which both in vertebrates and many invertebrates is one of the best known effects of the Mg ion. It should be remembered that the Mg ion is known to decrease permeability. It seems suggestive that the average oxygen consumption in the $MgCl_2$ solution was only very slightly higher than that found in a pure NaCl solution, the difference may well be within the limits of experimental error. The results of these experiments are summarized in Figure 5 and Figure 6. The stimulating effect on oxygen consumption is represented by the following two series:

Cations: Na = or slightly $<$ Mg $<$ Ca = NH_4 $<$ K.
Anions: Cl = or slightly $<$ SO_4 $<$ NO_2 = NO_3 $<$ PO_4.

The increase in oxygen consumption was especially pronounced in the KCl solution, the rate being about twice that found in an isotonic NaCl solution. This stimulating effect of the K ion is in line with experiments reported by other investigators on a variety of objects (literature in Heilbrunn, 1937).

SUMMARY

1. The temperature range tolerated by a larval Eustrongylides is great. The worms were not harmed by temperatures between 5° C. and 45° C., but 48° C. proved to be injurious.

2. The oxygen consumption was studied in this temperature range. Its increase with rising temperature could be expressed by two lines only, if the Q_{10} was calculated or if Arrhenius' formula was used. A fairly good fit to a single line resulted however if Bělehrádek's formula was applied.

3. The oxygen consumption remained practically unchanged in the pH range 3.4 to 8.3, but it was increased in the pH ranges 1.1 to 2 and 9 to 10.7. It is possible that the increase in the extreme acid range is of biological significance, while that in the extreme alkaline range is probably only a reaction to unfavorable environmental conditions.

4. The oxygen consumption was studied in a series of isotonic solutions of various inorganic substances. Of all the solutions tested, only $NaNO_2$ and to a lesser degree KCl were definitely toxic. The oxygen consumption was stimulated by various ions according to the following series:

Cations: Na = or slightly $<$ Mg $<$ Ca = NH_4 $<$ K.
Anions: Cl = or slightly $<$ SO_4 $<$ NO_2 = NO_3 $<$ PO_4.

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FURTHER EXPERIMENTS ON CELLULOSE DIGESTION BY THE PROTOZOA IN THE RUMEN OF CATTLE

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Studies on *Diplodinium* (*Eudiplodinium*) *neglectum* Dogiel have shown that this rumen protozoan may be grown continuously *in vitro* and that it possesses the capacity to digest cellulose (Hungate, 1942). Since the various species of rumen protozoa may differ in their ability to digest cellulose it has seemed desirable to extend the investigation to additional forms before drawing any general conclusions on the role played by the protozoa in the nutrition of their host.

By employing modifications of the culture technique used with *D. neglectum* it has been possible to grow *D.* (*Eudiplodinium*) *maggii*, *D.* (*Polyplastron*) *multivesciculatum*, *D.* (*Anoplodinium*) *denticulatum*, and *Entodinium caudatum*. These forms have been identified with the aid of Dogiel's monograph (1927). They correspond fairly closely to his descriptions, with one exception which is noted below.

EXPERIMENTS WITH DIPLODINIUM MAGGII

Cultures of *D. maggii* were obtained by inoculating fresh rumen contents into 40 milliliters of a balanced salt solution (Hungate, 1942) containing 30 milligrams of dried ground grass (*Lolium italicum*). The cultures were incubated at 39° C. under anaerobic conditions. *D. maggii*, *D. neglectum*, and a few Entodinium survived and grew in this medium.

One-half of each culture was transferred every 48 hours in order to prevent accumulation of staling products. To the transferred portion were added 20 milliliters of fresh salt solution and 15 milligrams of grass. A small sample (0.1 to 0.5 milliliter) was regularly removed and examined microscopically as a means of following growth of the protozoa and the suitability of the culture methods employed. The 48-hour interval between transfers was soon found to be too long. The protozoa appeared sluggish just before transfer and occasionally dead ones were seen. This was prevented by decreasing the time between transfers to 24 hours.

Clone cultures were obtained by isolating single individuals in 5 milliliters of salt solution plus grass. Special anaerobic vessels permitting frequent observations through a binocular dissecting microscope were used. In six clones the protozoa were counted three days after isolation and counts of 5, 4, 9, 8, 10, and 8, respectively, were obtained. The grass particles tended to hide some individuals and so the count was probably slightly less than the number actually present. The number seen is about that to be expected if the division rate is of the order of magnitude of once per day. This rate is also suggested by the fact that the concentration of individuals in the cultures remained approximately constant even though in transferring they were diluted daily with an equal volume of fresh medium.

D. maggii has been carried in the laboratory as a mixed protozoan culture for two months and as a clone culture for three more months. The concentration of individuals at the time of transfer has fluctuated between 10 and 100 per milliliter during most of the culture period. After the clone culture had been carried for three months the number of individuals decreased to only one or two per milliliter and the culture was discontinued.

During the period when the concentration of protozoa was fairly high the clone was used for experiments on cellulose digestion by extracts prepared from the protozoa. The number of flask cultures was increased to 32. Sixteen of these supplied protozoa for cellulase tests whereas the others were transferred.

The flasks supplying protozoa for the extracts received at the last transfer a small amount of a suspension of finely divided cellulose in addition to the grass. The gas produced by fermentation of the cellulose carried both cellulose and grass to the top of the liquid medium. The protozoa collected on the bottom and could be pipetted off without disturbing the surface cap. They were strained through bolting silk, washed, and allowed to settle in a large volume of the balanced salt solution. By these manipulations the protozoa were separated from most of the grass particles. They were either used immediately for enzyme extracts or if it was desired to subject them first to a period of starvation (see below) they were transferred to 50 milliliters of fresh inorganic solution in another flask and left for a time without food.

TABLE I

Results of cellulase experiments with D. maggii

Experiment	Starvation period	Protozoan extract plus cellulose	Protozoan extract, no cellulose	Boiled protozoan extract plus cellulose	Extract of debris plus cellulose	Boiled extract of debris plus cellulose
1	4 hours	+++*		±		
2	4 hours	+++	±		—	—
3	12 hours	++	±			

* +'s indicate the approximate magnitude of the reduction; ± represents only a trace of reducing material; — shows that no reducing material was formed.

Extracts from the protozoa were tested for their cellulolytic activity at a pH of 5.8. Reduction of Benedict's solution served to measure the sugar formed. In the first experiment there was almost as much reducing material formed in a sample of the extract alone as in the extract plus cellulose. In subsequent experiments the protozoa were starved for a short time before extraction. With this precaution it was possible to decrease the amount of reduction due to the extract itself.

The results of several experiments are shown in Table I. The number of plus signs indicates the magnitude of the reduction observed.

These experiments provide positive evidence of a cellulase in *D. maggii*. Since extracts of the debris in the culture (partially decomposed grass and cellulose containing numerous bacteria) show no cellulolytic action it must be concluded that the demonstrated cellulase is elaborated within the bodies of the protozoa. It cannot be ascribed to the cellulose-decomposing bacteria which are present in the culture.

The ease with which reserve carbohydrates can be demonstrated microscopically in Diplodinium (Schulze, 1924; Trier, 1926) suggested another approach to the problem of cellulose digestion. A batch of *D. maggii* was washed and then starved. After seven hours of starvation and again after 11 hours a small sample was removed and stained with iodine. A photomicrograph of the stained 7-hour sample is shown in Figure 1a and of the 11-hour sample in Figure 1b. The paraglycogen appears black in the figures.

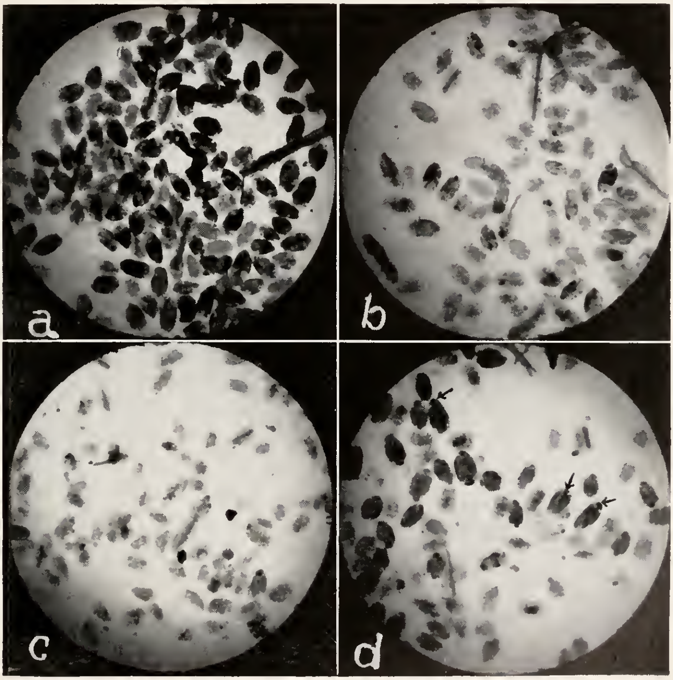

FIGURE 1. (a) *D. maggii*, starved seven hours and then stained with iodine; (b) starved 11 hours; (c) starved 13 hours; (d) starved 11 hours, then fed cellulose and photographed after two more hours.

It is evident from the figure that most of the food reserves are depleted after 11 hours, suggesting rather high metabolic requirements. The protozoa are not entirely uniform in this respect, however. Some still contain reserve food, due either to greater initial stores or to feeding on the few grass particles not removed by washing. Others appear entirely devoid of paraglycogen though still living. Some succumbed during the starvation period, as found by microscopic examination of an unstained sample.

At the same time that the sample pictured in Figure 1b was obtained two others were also removed. One was placed in a small tube of inorganic solution and the other was similarly treated except that a small amount of a fine cellulose suspension was added. The cellulose was almost instantly ingested by most of the living protozoa. After two hours of incubation at 39° C. the protozoa were removed from each of the tubes, stained with iodine, and photographed. The results are shown in Figure 1c for the protozoa receiving no cellulose and in 1d for those with cellulose. The ingested cellulose itself gives a dark appearance to the central portions of the fed animals and this should not be confused with the paraglycogen which was deposited. The paraglycogen is present in the tips of the cells (indicated by arrows in Figure 1d) whereas the cellulose is in the large central digestive sack. The individuals which show no cellulose or paraglycogen are those which had succumbed to starvation. In the sample without cellulose (Figure 1c) most of the cells are devoid of paraglycogen, in marked contrast to the fed.

This experiment shows the rapidity with which cellulose is digested and assimilated by *D. maggii*. Bacteria could hardly exert any significant digestive action on the cellulose during the short time (two hours) between its addition and the appearance of reserves in the protozoa, particularly when the thorough washing of the protozoa is recalled. Thus, these observations fully support the conclusion that *D. maggii* forms a cellulase.

Experiments on Diplodinium multivesciculatum

This interesting species shows numerous contractile vacuoles instead of the usual two. It also has two narrow skeletal plates on the right side and suggestions of skeletal structures on the left. The specimens cultured resembled those described by Dogiel (1927) with the exception that the skeletal plates on the left side were not as definite as in his description. They seemed to be influenced by the state of nutrition of the protozoa and were less apparent in poorly fed individuals.

D. multivesciculatum was found in cultures containing grass and cellulose and also in those containing grass and starch. Later it was discovered that best growth occurred with grass, cellulose, and ground wheat. This medium was used to grow the protozoa during most of the period (100 days) in which the laboratory culture was maintained.

Growth of *D. multivesciculatum* was slower than that of *maggii* or *neglectum* and the concentration of the protozoa could be maintained only when 2-day transfers were made. This indicates a division rate of once in 48 hours. The concentration was around 100 individuals per milliliter under the most favorable conditions.

D. multivesciculatum seemed to be more sensitive to environmental changes than were any of the other protozoa studied. Individuals of most species showed normal activity for some time after being removed from the culture and placed in a depression slide for microscopic examination. In the case of *multivesciculatum* only rarely was a motile individual observed even when examined immediately after removal from the culture.

Several attempts to obtain clone cultures were unsuccessful, presumably due to sensitivity to handling. However, *multivesciculatum* seemed to be fairly resistant to high acidity and cultures containing it as only the large protozoan could be obtained from old flasks. *Entodinium caudatum* was the only other species of protozoa present.

In order to test for cellulase the protozoa were raised in large numbers and freed of Entodinium and debris by straining through suitable meshes of bolting silk and by further washing. Extracts were prepared and gave positive tests for cellulase. In the cultures containing grass, cellulose, and wheat it was the cellulose that made up most of the material in the digestive sack.

EXPERIMENTS ON DIPLODINIUM DENTICULATUM

Protozoa of this species appeared in cultures containing cellulose and grass. Growth occurred also when ground wheat was added to this medium. Maintenance of numbers during daily transfers indicated that the division rate was at least once every 24 hours. The concentration of individuals varied between 100 and 300 per milliliter.

A clone culture was obtained starting with an individual showing the six caudal spines characteristic of the species. After several weeks the descendants of this individual were of many morphological types. Individuals with no spines, with two poorly developed ones, and with several rudimentary spines were seen. No individuals with the six typical spines were found in the culture at this time. These observations fully substantiate Poljansky and Strelkow's report (1934) on variation in this form. After two months the clone died in spite of efforts to maintain suitable conditions.

Because of the small size no attempt was made to grow *D. denticulatum* in sufficient numbers to test extracts for cellulase. However, it is probable that it resembles *D. maggii*, *multivesciculatum*, and *neglectum* in being able to utilize this material. Cellulose is required in the culture medium and large quantities of it are ingested.

EXPERIMENTS ON ENTODINIUM CAUDATUM

Protozoa belonging to this genus were observed in most of the cultures inoculated with rumen contents and transferred at daily intervals. Only a small number were in the cultures containing grass or grass plus cellulose and they seemed to ingest very little of these substrates. When soluble starch was added they became more numerous and with ground wheat the concentration increased to 2000 to 5000 per milliliter. The starch and wheat were ingested to some extent but ingestion of large particles was not nearly as striking as in Diplodinium. In some cases food vacuoles filled with bacteria were seen.

Individuals resembling *E. simplex*, *E. longispinum*, and *E. caudatum* were observed in the cultures. An individual classified as *E. simplex* was inoculated singly into a small amount of medium and a clone was obtained from it. After two weeks the members of the clone were examined microscopically and individuals similar to *simplex*, *longispinum*, and *caudatum* were observed. This is again in entire agreement with the observations of Poljansky and Strelkow on this form and indicates that *simplex* and *longispinum* are synonymous with *caudatum*.

In contrast to Diplodinium, grass and cellulose could be omitted from the culture medium for *E. caudatum* when wheat was used as food. Omission of the cellulose did not affect the concentration of the protozoa, but omission of the grass resulted in some decrease in numbers.

The apparent inability of *E. caudatum* to use cellulose in the cultures suggested that cellulase was lacking. This was found to be the case when extracts were tested. No cellulase could be demonstrated even though the extracts were more concentrated than those giving positive tests in the case of Diplodinium.

CELLULOSE DIGESTION BY OTHER RUMEN PROTOZOA

The species of rumen protozoa reared thus far in the laboratory constitute only a small fraction of those inhabiting the rumen. However, the demonstration that Diplodinium species digest cellulose and that *Entodinium caudatum* does not, makes it possible to draw some fairly satisfactory conclusions regarding most of the other rumen protozoa.

Species of Diplodinium resemble each other in their habit of ingesting large quantities of cellulosic plant materials. Out of four species of Diplodinium studied, a cellulase has been found in three and all evidence points to its presence in the fourth. The habit of ingesting large quantities of cellulosic materials thus seems to be accompanied by an ability to digest cellulose. *Entodinium caudatum* ingests very little cellulose and forms no cellulolytic enzyme. This correlation between the presence of cellulase and ingestion of cellulose probably holds also for the other rumen protozoa. Species ingesting large quantities of large plant particles digest cellulose whereas others do not. Their capacity in this respect can be ascertained by a direct microscopic examination of fresh rumen contents.

Among the rumen protozoa which have been examined during the present investigation only species of Diplodinium have been observed to ingest large quantities of plant materials. The other protozoa studied have included species of Entodinium, Isotricha, Dasytricha, and Bütschlia and in none of them have plant parts been observed to be ingested in an amount comparable to that in Diplodinium. It is unlikely that any of them digest cellulose.

The question of the utility of the rumen protoa to their host has hinged largely on whether they digest cellulose (Becker, Schulz and Emmerson, 1929). The ruminant itself produces no cellulase and its utilization of cellulose is thus dependent upon the microorganisms in the rumen. Those which digest cellulose aid the ruminant and may be classified as symbionts, whereas others are either slightly harmful or at best merely commensals. On this basis the various species of Diplodinium may be regarded as symbionts but Entodinium, Isotricha, Dasytricha, and Bütschlia are not.

SUMMARY

Diplodinium maggii, *D. multivesciculatum*, *D. denticulatum*, and *Entodinium caudatum* can be grown in flask cultures using as substrates grass, cellulose, and ground wheat, either singly or in combination, depending on the species being cultured.

Clone cultures of *D. denticulatum* and *E. caudatum* show wide variations in the morphology of individuals in the clone.

A rapid synthesis of food reserves from cellulose has been demonstrated in *D. maggii*.

The results of cultural studies and of experiments on cellulose digestion by extracts of the protozoa show that the three species of Diplodinium digest cel-

lulose but *E. caudatum* does not. Microscopic observations indicate that all species of Diplodinium digest cellulose, whereas Entodinium, Isotricha, Dasytricha, and Bütschlia do not. Thus, Diplodinium is the only protozoan which may be considered a symbiont.

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METHYLENE BLUE, POTASSIUM CYANIDE AND CARBON MONOXIDE AS INDICATORS FOR STUDYING THE OXIDATION-REDUCTION POTENTIALS OF DEVELOPING MARINE EGGS

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The effects of inhibitors and accelerators of oxidations in marine eggs have been studied first by Warburg (1910) using sea urchin eggs. Rünnstrom (1930) made a detailed study of the effects of carbon monoxide, potassium cyanide, and methylene blue. Later on this was studied by Clowes and Krahl (1940). They used two stages of sea urchin eggs, unfertilized and fertilized. The present experiments[1] extend this work to further stages of *Arbacia punctulata* and *Asterias forbesii*, and measure the effects of the mentioned reagents on their oxygen consumption and development.

METHODS

Materials. Eight of these stages of *Arbacia punctulata* (Lam.) or *Asterias forbesii* (Desor.) were used: unfertilized and fertilized eggs, early cleavages, morula, blastula, early gastrula, late gastrula, and pluteus. The inhibitors of respiration were CO, 99.5 per cent pure,[2] and KCN, 5×10^{-4} M; and the accelerator, methylene blue, .002 percent. These concentrations were higher than those used by Rünnstrom and therefore caused more pronounced effects.

Procedure. The experiments were done at Woods Hole, Massachusetts, from June to August, 1941. The oxygen consumption of a stated volume of eggs or larvae was measured in cubic millimeters per hour, according to the Barcroft-Warburg method, in a standard set. The technique used was that described by Dixon (1934). The vessels were standard conical type with wells inside to hold the KOH (.2 co. of 20 per cent KOH) for absorption of CO_2 and a side-arm or onset which contained the KCN or methylene blue solution, or sea water to be added at the expiration of the control readings. The eggs were collected by the methods recommended by E. B. Harvey and centrifuged at about 3000X gravity for one minute to give a concentrated suspension of loosely-packed eggs. They were not tightly packed, to avoid injury. Subsequent fertilization tests showed that 95 to 100 per cent fertilization membranes and normal cleavage were obtained. Four cc. of this suspension so obtained was diluted with sea water to a total volume of 30 cc. From this suspension 2 cc. was placed in each of the 12 Warburg vessels, two vessels being reserved for controls, lacking eggs. These twelve vessels were used for the measurement of oxygen consumption of developing eggs, including the vessels being used for normal control of eggs in sea water. The following conditions were established in one or another of the experiments:

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[1] In a preliminary report (Brooks, 1941) lower concentrations of KCN and methylene blue were used, viz., 2.5×10^{-4} M and 1.2×10^{-4} per cent. These concentrations were nearer the optimum value for antagonism experiments.

[2] Carbon monoxide was generated in the usual way by the reaction between formic acid and boiling concentrated H_2SO_4. This generator had been previously operated for several hours, and the gas was led directly by way of a condensor through soda lime to the Warburg respiration chamber. It was vented through the three-way cock at the top of the manometer, the Brody solution in the manometer forced up to the cock and then allowed to settle back to the mark (20). The direction of the flow was reversed several times, and the Brody solution allowed to fall to the zero mark again under flowing CO after being forced up to the cock. The gas consumption in the CO experiments, occurring principally in the first hour, suggests that air was trapped in dead spaces or that oxygen diffused into the system through the rubber connections as the CO was being led into the manometer. The amount of gas consumption in subsequent experiments indicates that not more than 0.5 per cent O_2 was present in the vessels. An analysis of the method was made by Dr. W. B. Amberson by means of the Haldane method. This showed that there was 0.5 per cent O_2 present in the sample tested.

1. Control eggs in sea water, as above, always used.
2. Eggs in sea water to which methylene blue, (.002 per cent) was added.[3]
3. Eggs in sea water in an atmosphere of CO (99.5 per cent).
4. Eggs in sea water to which KCN (5×10^{-4} M) was added.
5. Eggs in sea water with atmosphere of CO or in sea water plus KCN to which methylene blue (.002 per cent) was added.

To produce these conditions two procedures were involved: *a*) For KCN and methylene blue .5 cc. of a solution was placed in the side-arm of the vessel in such a concentration as to give the desired concentration when mixed with the eggs. This admixture was added without opening the vessels following a period of one hour in which the oxygen consumption had been measured. *b*) Warburg vessels in which CO was used were filled with CO at atmospheric pressure by prolonged passage of freshly generated CO. Comparisons were instituted between the oxygen consumption formed in these with and without subsequent addition of methylene blue, and between these, CO-filled vessels and simultaneously air-filled vessels. The two vessels lacking eggs contained:

6. Vessels without eggs, but containing sea water.
7. Barometric control.

No attempt was made to measure the number of eggs used, as these experiments were not designed for absolute measurements of single eggs, but of the *relative changes in the rates of approximately equal numbers of eggs as affected* by the listed reagents.

To make the procedure more comprehensible the details of a typical experiment are here given. There were 14 manometers in all, run simultaneously. Each set as listed in Tables I and II consisted of four different combinations in triplicate, plus the two controls listed above (No. 6 and No. 7). For example, there were three vessels in which eggs and sea water alone were used (controls); three vessels in which eggs in sea water with an atmosphere of CO were used; three vessels in which eggs in sea water to which methylene blue was added later; and three vessels in which eggs in sea water with an atmosphere of CO were used

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[3] Fresh chemicals for each experiment were used. To dissolve methylene blue in sea water, it was rubbed up in a few drops of distilled water and smoothed to a paste before adding the sea water. The desired concentration was then made from this concentrated solution by adding sea water. In this way the error caused by adding a few drops of distilled water became negligible.

Table I

Effect of methylene blue, CO and KCN in various combinations upon the oxygen consumption of different stages of development of *Asterias forbesii* and *Arbacia punctulata*. In each set there are four combinations in triplicate (in addition to the two controls). Columns 3 to 9 represent the average cmm. O_2 consumed per hour in each set. Probable error is less than 5 per cent of the mean. Concentration of KCN is 5×10^{-4} M; methylene blue, .002 per cent; CO, 99.5 per cent, temperature 20°; pH, 8.05; shaking by 50–60 excursions per minute of 6 cm. amplitude; volume of eggs presumably between 0.10 and 0.15 cc. in 2 cc. total per vessel.

		In air		In CO		In air		In CO
Number of experiments	Number of sets	Without methylene blue	With methylene blue	Without methylene blue	With methylene blue	+KCN	KCN +methylene blue	+KCN
		cubic mm. of O_2 per hour		cubic mm. of O_2 per hour		cubic mm. of O_2 per hour		
1.	2.	3.	4.	5.	6.	7.	8.	9.
Immature eggs, Asterias								
I.	2	14	—	6.1	—	7	—	3.6
Unfertilized eggs, Asterias								
II.	2	20	—	10	—	18	—	6
III.	5	20	40	8	9	—	—	—
Fertilized eggs, Asterias								
IV.	2	42	58	4.5	5	—	—	—
Unfertilized eggs, Arbacia								
V.	6	12	22	9	10	—	—	—
Early cleavages, Arbacia								
VI.	3	36	42	—	—	6	8	—
VII.	4	26	28	5	6	—	—	—
Morula, Arbacia								
VIII.	3	27	33	8	8	—	—	—
Blastula, Arbacia								
IX.	2	35	43	—	—	5	15	—
X.	3	24	29	3	6	—	—	—
Early gastrula, Arbacia								
XI.	2	28	28	—	—	1.0	2.5	—
XII.	6	86	87	4	4	—	—	—
Late gastrula, Arbacia								
XIII.	4	30	30	4	4	—	—	—
Pluteus, Arbacia								
XIV.	3	18	25	—	—	3	9	—
XV.	4	21	28	2	5	—	—	—

TABLE II

The relative effects of various reagents on eggs and larvae of *Arbacia punctulata* and *Asterias forbesii* recalculated from *Table I*. Columns A to E represent ratios ×100 of rates of O_2 consumption in the different experiments. Column A: the rate in the presence of methylene blue as compared with its absence; Column B: methylene blue and CO as compared with CO alone; Column C: KCN as compared with the control; Column D: KCN and methylene blue as compared with KCN; Column E: CO as compared with the control. Roman numerals refer to experiments in *Table I*, representing different stages of development from immature eggs to pluteus. Concentration of KCN, 5×10^{-4} M; methylene blue, .002 per cent; CO, 99.5 per cent.

Ratios	A	B	C	D	E
	$\frac{\text{M.B.}}{\text{Control}}$	$\frac{\text{M.B.-CO}}{\text{CO}}$	$\frac{\text{KCN}}{\text{Control}}$	$\frac{\text{KCN-M.B.}}{\text{KCN}}$	$\frac{\text{CO}}{\text{Control}}$
Number of experiments	Per cent	Per cent	Per cent	Per cent	Per cent
Immature eggs, Asterias					
I.	—	—	50	—	42
Unfertilized eggs, Asterias					
II.	—	—	90	—	50
III.	200	112	—	—	40
Fertilized eggs, Asterias					
IV.	138	111	—	—	10
Unfertilized eggs, Arbacia					
V.	183	111	92	—	75
Early cleavages, Arbacia					
VI.	116	—	16	133	—
VII.	117	120	—	—	19
Morula, Arbacia					
VIII.	122	100	—	—	29
Blastula, Arbacia					
IX.	122	—	14	300	—
X.	120	200	—	—	12
Early gastrula, Arbacia					
XI.	100	—	3	250	—
XII.	100	100	—	—	4
Late gastrula, Arbacia					
XIII.	100	100	—	—	13
Pluteus, Arbacia					
XIV.	138	—	16	300	—
XV.	133	250	—	—	9

to which methylene blue was added later. This, plus the two controls listed above, makes 14 vessels. In this way all the combinations necessary for one experiment were done in triplicate with samples from the same suspension, at the same time, with the same temperature and at the same rate of shaking. The composition of the sets varied with the reagents used, but *there was always a control for eggs or larvae in sea water, for sea water without eggs and for barometric pressure.* In fact the experiments were doubly controlled: 1) Readings were taken for one hour before the reagents were added from the onsets, so that the contents of each separate manometer and vessel were read with and without the addition of the experimental solution. 2) In addition to this, manometers and vessels were set up containing eggs and sea water only, and onsets containing sea water only which was added at the same time the experimental solutions were added.

The *temperature* was kept at 20.0 ± .1° for all experiments.

In a few of the cyanide experiments, the Krebs method was used to equalize the HCN pressure for the concentration of KCN used. No difference could be found between the results obtained with or without this modification.

The *rate of shaking* of the manometers was about 50 to 60 round trips per minute at an amplitude of 6 cm. This was found not to cause any injury to the eggs as shown by subsequent tests on eggs fertilized after shaking. These produced between 95 and 100 per cent development, which was the same as the per cent development in the unshaken eggs. This rate of shaking also allowed constant oxygen consumption for four hours or more. The experiments in most cases were run not more than three hours. In all the experiments, the rate of shaking was sufficient to keep the eggs evenly distributed throughout the solution, but not enough to injure them as shown by tests of fertilizability.

In measuring oxygen consumption in air, the rate during the first hour was taken as the basis. At this point the contents of the onsets were added and the measurements continued for one to two hours more or even longer. Readings were taken every 10 minutes and the change in the slope of the curves so obtained showed whether or not an effect was produced. The number of cmm. of O_2 consumed during intervals of ten minutes was calculated according to Warburg's equation (1924). Columns 3 to 9 of each table give the average number of cmm. of oxygen per hour for each set of experiments.

The measurement of gas consumption, or removal when CO was involved, involves other bases which are referred to below.

The *probable error* of the mean was less than 5 per cent of the mean in each case.

RESULTS

Effects on the rate of oxygen consumption

The striking differences between the oxygen consumption by unfertilized eggs and by fertilized eggs is well known (Warburg, 1910). The differences in rate in the various stages used in the writer's experiments are also significant. These will be discussed under the separate headings below (Tables I and II).

Table I gives the figures for the number of cubic millimeters of O_2 consumed by the eggs. The figures are the averages of several similar experiments, their numbers being given in column 2. Since each set was done in triplicate these figures are to be multiplied by three to indicate the number of separate experiments done.

Table II is a summary and compilation of the effects of the various reagents. For example, under "A," methylene blue raises the rate of O_2 consumption of unfertilized Asterias eggs to 200 per cent of the control. Column "C" shows that KCN depresses the rate of O_2 consumption of early gastrulas to 3 per cent of that of the control. Column "D" shows that KCN and methylene blue acting together allow a rate of O_2 consumption in early cleavages in Arbacia which is 133 per cent of that found when KCN is used alone. The Roman numerals refer to experiments in Table I, and represent different stages of development.

Effects of methylene blue. This dye accelerates oxygen consumption. Unfertilized Asterias eggs showed the greatest acceleration, the rate of O_2 consumption reaching 200 per cent of the controls, while unfertilized Arbacia eggs reached 185 per cent of the rate of the controls. After fertilization, the rate of O_2 consumption, with methylene blue dropped to 138 per cent of that of the controls in Asterias, and 116 per cent of that of the controls in Arbacia. During the morula and blastula stages in Arbacia the rate of O_2 consumption with methylene blue became 122 per cent of that of the controls. There was no effect in the early and late gastrula stages but methylene blue increases to 138 per cent in the pluteus stage.

Effects of potassium cyanide. Three concentrations of KCN were tried: (1), 5×10^{-4} (the concentration represented in the tables), (2), 1×10^{-3}, and (3), 1×10^{-5}. In the first two concentrations, the rate of O_2 consumption in unfertilized eggs was slightly decreased (See table for (1)) but the third concentration produced either no change or an increase. Only (1) was used here in detail. After fertilization, KCN (5×10^{-4} M) produces a considerable decrease in the rate of O_2 consumption in all the forms, but this decrease is most pronounced in the gastrula stage where it falls to 3 per cent of the controls. In the early cleavage states the rate of O_2 consumption with KCN is 16 per cent, in the blastula, 14 per cent, and in the pluteus, 16 per cent of the controls. In immature Asterias eggs, the rate of O_2 consumption was decreased by KCN to 50 per cent of that of the controls.

Effects of carbon monoxide. Where a low concentration of oxygen is used together with approximately one atmosphere of CO, a normal rate of respiration might be due to an adequate supply of oxygen. Amberson (1928) found that the critical oxygen tension for marine eggs is from 5 to 3.5 per cent. Below this the eggs rapidly cease respiring. Rünstrom's experiments (1930) were done above this range of O_2 tension, so that in this case it may well be that there was enough oxygen present to supply the eggs. In the writer's experiments, CO, 99.5 per cent pure[2] was used; here simultaneous runs were done with air and CO-filled Warburg vessels. The former was taken as the control. Carbon monoxide depressed the respiration to such an extent that after a few hours respiration ceased entirely. The effects, even so, showed that respiration did not stop instantly. The gastrula stage seemed to be the most sensitive, shown by cessation of gas consumption before the other stages. The least effect was in the unfertilized eggs. For example in column E, Table II, No. XII, the rate with

CO falls to 4 per cent of that of the controls; where as in unfertilized Arbacia, it is 75 per cent. In other stages the rates are higher than in the early gastrula.

It is felt that this small absorption of gas may represent oxygen consumption supported by the traces of oxygen present in the CO atmosphere. The experiments by Loeb (1895) on the effects of hydrogen and nitrogen atmospheres on echinoderm eggs and larvae give the same picture as these experiments with CO. It is suggested that the effects of CO may result from the exclusion of oxygen, thereby eliminating the oxidized forms of the enzymes.

Antagonism between methylene blue and inhibitors. When methylene blue was added to eggs or larvae in an atmosphere of CO, there was an increase in the rate of O_2 consumption. However, in the concentration used here, (0.002 per cent) the maximum antagonistic action was not obtained. The antagonism between methylene blue and carbon monoxide in optimum concentrations has been discussed elsewhere (Brooks, 1935, 1941).

Additive effect of cyanide and carbon monoxide.—When cyanide and CO were used together, the rate of uptake of gas was even less than when either was used alone. Only immature and unfertilized Asterias eggs were used here (see Table I, Column 9).

RESULTS

Effects on Development and Survival

After the measurements had been made on oxygen consumption, the eggs or embryos were taken out of the manometer vessels and samples of them returned to sea water. To do this, the eggs in the vessels were lightly swirled to give an even suspension, after which .1 cc. of egg suspension was withdrawn and discharged into 200 cc. of fresh sea water in 15 cm. finger bowls. Development occurred at the temperature of the running sea water in the laboratory, 16 to 22° C. depending upon the month. About one cmm. of dry sperm was suspended in 200 cc. of sea water, well mixed, and one drop of this suspension added to each finger bowl. After the eggs had settled, the sea water was renewed to get rid of the sperm. The development of about 100 eggs per sample was followed.

Effects of methylene blue. Methylene blue, 0.002 per cent in sea water, had acted upon the eggs for a time between two and three hours. Fertilization of unfertilized eggs gave success equal to or better than that found in untreated eggs (controls). During the main spawning season this was between 80 and 100 per cent formation of fertilization membranes, but at the beginning of the season, this was much lower (from 10 to 50 per cent): the controls gave as low as or lower percentages of success than the methylene blue-treated eggs.

Development was accelerated by methylene blue; when the earlier stages were concerned, it was noted that many blastulae were produced among the treated individuals in the time in which only a few were found in the controls.

The plutei produced from eggs or from the earlier developmental stages were measured and the largest ten to 15 in each sample measured. It was found that the average of the total lengths of the controls was 280 μ as compared with 420 μ in the methylene blue-treated samples. Embryos experimented upon in the gastrula stage gave plutei without arms (controls), in the time in which the methylene blue-treated ones developed large arms. The pluteus stage was still active at the end of nine days while the controls survived only four days. It may be noted that in toxic concentrations, methylene blue accelerates the cytolysis of unfertilized eggs and to a less extent that of fertilized eggs.

Effects of carbon monoxide, with and without methylene blue. In these experiments eggs or embryos were exposed to CO (99.5 per cent pure) alone, or together with 0.002 per cent methylene blue, for a period of time between two and three hours. Experimental and control lots were then returned to sea water as described above. In contrast with cyanide which allowed substantial recovery, CO alone stopped movement of the embryos irreversibly, and induced subsequent cytolysis. Unfertilized eggs treated with CO alone lost the capacity to divide when they were subsequently fertilized. The addition of methylene blue acting simultaneously with CO, restored the capacity for subsequent fertilization. Fertilized eggs treated with CO alone lost the power to cleave in the recovery period and were cytolyzed. Addition of methylene blue was ineffective. Carbon monoxide alone permanently stopped the motion of gastrulae and plutei, and led to cytolysis within 24 hours. Addition of methylene blue failed to restore motility, but it did prevent cytolysis during at least the 48 hours of observation.

Effects on staining and reduction of methylene blue. Living eggs in methylene blue alone showed stained granules in the cytoplasm but the nucleus and the cytoplasmic matrix were not visibly stained. The only exception to this was in the case of cyanide and methylene blue experiments where the nucleus was stained in addition to the granules. Since these were still swimming, they could not be considered to be dead.

When methylene blue was used in conjunction with carbon monoxide, the dye was presumably reduced since no color remained in either the cells or in the surrounding solution while the manometer vessels were closed. Methylene blue is not reduced in an atmosphere of carbon monoxide or in the absence of oxygen, in the absence of living cells. Living cells must therefore furnish the hydrogen for the reduction of the dye.

Discussion

In the past it has been customary to account for the fact that cyanide fails to block oxygen consumption of many types of cells beyond a minimum by inferring that there exists a "cyanide-resistant" respiration, in addition to the "cyanide-sensitive" respiration. Many cases of "cyanide-resistant" animals or plants have been reported. For example, Lund (1918) found that cyanide does not affect the respiration of Paramoecium; Shoup and Boykin (1931), confirmed this in detail; Pett (1936), showed that a certain yeast is not affected by cyanide. Dixon and Elliott (1929), found that only a portion of the respiration of at least one strain of yeast is affected by cyanide. When cyanide does have an inhibitory effect as shown for rat liver by Dixon and Elliott (1929), this could be completely reversed by quickly rinsing the tissue with buffer solution, even when the concentration of cyanide was M/30. A resumé of some cyanide-resistant tissues is given by Dixon and Elliott. In some cases, activation is produced by cyanide. This has been shown by Reynolds (1924) in the case of Fusarium; by Hanes and Barker (1931) for potato tubers; by Tomkins (1932) for moulds; by Watanabe (1932) for certain algae; and by Kisch (1933) in the case of certain mammalian tissues. An interesting point is that the formation of flavin in yeast is activated by cyanide as shown by Pett (1936).

The extent of this cyanide respiration suggested to some an explanation on the basis of a reversible combination between CN^- and one enzyme according to Warburg's postulation, a reaction whose extent can be predicted with the use of a reaction constant. Ross (1938) has offered such a calculation on Nitella oxygen consumption. Fisher and Öhnell (1940) have developed this idea in greater detail.

However, since cyanide gives different effects not only in a wide variety of tissues but also in the different stages of the developing echinoderm eggs and embryos, it is felt that these results can be explained on a basis quite independent from the postulation of several different enzyme systems, each with its combination constant.

The interpretation offered by the writer is based upon the relation between the rate of oxygen consumption and the redox potential surrounding the living cells (Heymans and Heymans, 1922; Genevois, 1928; Barron, 1930). In developing this theory, the terminology of Clark (1927) is used. According to this, the redox potentials (E_h values at pH 7.0) for observed aerobic cells were found to be not far from 0.0 volts (Needham and Needham, 1925; Brooks, 1926). The workers mentioned above found that as the redox potential becomes more positive, within certain limits of E_h and pH, the oxygen consumption increases.

When methylene blue acts on living tissues whose E_h is less than that of the methylene blue, the poising influence of methylene blue tends to raise the E_h toward that of methylene blue. The couple, methylene blue: leuco methylene blue, has an E_0 of around 0.0 volts at pH 7.0. The oxidized form, methylene blue, has been added in these experiments, and should poise the solution and eggs toward positive levels. It is logical to conclude that a rise in redox potential induced by this dye is due to this poising effect of the dye. At a constant concentration of the dye, this rise in E_h would increase with increasing disparity between the E_h level of the dye and that of the living cell.

Conversely, if there is no effect of methylene blue, when added to suspensions of living cells, one may well conclude that the E_h of the cells is at the same level as that of the dye solution itself. Therefore, we may essay an explanation on the basis that the E_h levels of living cells are shown by the magnitude of the effect of methylene blue on their oxygen consumption.

The following deductions are accordingly made on this basis. Unfertilized Asterias and Arbacia eggs have a lower redox potential than fertilized ones, since methylene blue produces a pronounced increase in the rate of O_2 consumption. The redox potential of the larvae in the early stages after fertilization is below optimum but rises in the gastrula stage to optimum. Here, there is no effect of methylene blue in these experiments. In the pluteus stage, there is again a decrease in the redox potential. It should be remembered that there must be an optimum E_h, above or below which a decrease in O_2 consumption occurs. A small change in E_h around the optimum must have practically no effect, while an equal change at more or less positive potentials would produce increasingly marked effects in oxygen consumption.

The lowered redox potential[4] of KCN, produced by its poising action in connection with an unknown oxidant (Barnard, 1933), rather than the reactions of a stable chemical combination of CN^- and the iron of the heme radical of the respiratory enzyme, can be used as a basis in interpreting the reduction of oxygen consumption caused by cyanide solutions. When there is no effect of KCN on the rate of oxygen consumption, it appears that the redox potential is already negative and is not lowered further by the addition of the reagent. Here the normal redox potential within the cells appears to be essentially the same as that which would be produced by KCN in the concentration used. This seems to be the case in unfertilized eggs. This conclusion agrees with the results with methylene blue, which show a large increase in the rate, indicating that methylene blue raises the potential to a considerable extent.

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[4] The work of Pett (1936) who found that cyanide increases the flavin content of yeast can be interpreted from the point of view of redox potentials; namely that the cyanide produced a more negative potential which was more favorable to the production of flavin.

It is also interesting that in the gastrula stage KCN reduces the rate of oxygen consumption to its lowest level, whereas methylene blue causes no change in the rate. It seems as though this stage has the highest redox potential of all those investigated. These experiments with KCN and methylene blue therefore appear significant in that KCN produces a lower potential, and the dye a higher one. This explanation has been offered previously by the writer (Brooks, 1935, 1941) to explain the action of methylene blue when cyanide is added to cells. It is merely a balancing between the two reagents to keep the redox potentials normal. Since different cells have different redox potentials, it will be necessary to use appropriate concentrations of KCN and methylene blue as determined by experimentation.

Here it is further suggested that these two reagents can be used as indicators of the redox potential of a cell. Since the cell's redox potential becomes more negative with increasing concentrations of KCN (within limits), one can ascertain the original redox potential by noting the effect of KCN on the rate of oxygen consumption. Conversely, methylene blue can be used to change the E_h in the opposite direction.

In these experiments no attempt was made to obtain the maximum antagonistic effects of methylene blue with KCN and CO. This has been done in other papers (Rünnstrom, 1930; Brooks, 1935). This paper shows merely the effects of adding the reagents in the concentration used in this paper. There is an antagonistic effect in each case, but optimum rate (Q_{O_2}) has not been sought.

In the system of oxidative and reductive enzymes in the egg and embryo of Arbacia and Asterias, the concept of the optimum must be applied. However it is necessary to assume that all of these enzymes must be in equally favorable states. For a given enzyme the highest rate of reaction, here thought of in terms of optimum oxygen consumption, occurs at the E_h where the reduced and oxidized states of this enzyne are equal in (active) concentration. One of the enzymes of such a system may be nearly oxidized and another at the same E_h may be nearly reduced. But at some optimum E_h, the best integration of their action must occur. This optimum is more nearly attained in fertilized eggs of these forms rather than in unfertilized eggs. It does not seem reasonable to assume that there are little or no respiratory enzyme systems in unfertilized eggs because they have a low rate of oxygen consumption. On the contrary, since methylene blue produces such a profound increase in the oxygen consumption, it would appear that this reagent poises the potential at an optimum where the activities of oxidants and reductants of the respiratory enzyme systems more nearly approximate each other. It would further appear that the process of fertilization in these eggs raises the redox potential to a more positive level which is closer to the optimum. Unless the unfertilized egg does not contain any respiratory enzyme (cytochrome oxidase), then any concentration of KCN should produce a decrease in the rate, if it is true that CN^- combines with the cytochrome oxidase as is postulated by Warburg (l.c.) and repeated by many others for example, Barron (1937), Krahl, Keltch, Neubeck and Clowes (1941), Henderson (1938), Wendel (1933, 1934). It seems preferable to think of these living cells of this nature as containing such an enzyme system, and the hypothesis of combination seems to be unessential.

The present experiments may be further tested in the light of additional evidence against the assumption that the effect of KCN on living cells is due to a combination of CN^- with the Fe of the heme of the cytochrome oxidase (respiratory enzyme), thereby inactivating it. This enzyme has not been isolated and there are no direct experiments to prove its existence. The evidence is derived from measurements of oxygen consumption. Those who favor the hypothesis that CN^- unites with the heme of the cytochrome oxidase, base their experimental evidence on chemically isolated compounds such as the hemoxhromogens and combinations of CN^- with these at high pH values, i.e., 9.0 to 13. (Hogness, Zscheile, Sidwell and Barron (1937), Barron (1937), Clark (1939), Clark, Taylor, Davies, and Vestling (1940). It appears quite evident, however, that such results at these *unphysiological* pH values can not be used to explain the conditions found in living cells. Theorell (1940) using cytochrome c (an oxidation link in most cells, which is also a heme compound and has been isolated), showed that CN^- forms combinations with this compound only in acid or alkaline solutions but not at pH values around 7.0 such as occur in living cells. This can be considered as further evidence against the hypothesis that CN^- combines with a heme such as the cytochrome oxidase which is supposed to exist in living cells.

A further observation made here can be understood on the basis of this concept: fertilized eggs to not cleave in CN^--containing media, but do form multiple asters without cell division. This suggests that division requires a higher redox potential, whereas aster formation goes on at lower redox potentials.

The effect of CO on the oxygen consumption of eggs and embryos of Asterias and Arbacia points to an interpretation which also obviates the assumption of combination of CO with an enzyme. Mere absence of oxygen suffices to explain these observed results. The fact that Theorell (1940) found that cytochrome c does not combine with CO at physiological pH ranges supports this.

It is of interest to recall in this connection the case of animals having hemoglobin. It is generally conceded that the principal effect of CO in stopping respiration is due to its affinity for hemoglobin, forming carbohemoglobin which is a stable compound. In proportion to this conversion the blood fails to carry oxygen to the tissues (Henderson, 1938). The effect of CO upon the oxidative enzyme is therefore secondary through the inactivation of the hemoglobin. If there is no hemoglobin present as in the case of marine eggs, one is led to conclude that in this case also the effect is due to lack of oxygen rather than to a specific effect such as combination of CO with the cytochrome oxydase. At the present writing it is not known whether there exists in Arbacia eggs a substance similar in action to the hemoglobin of mammals to combine with CO. The probability is, however, that the principal effect of CO on these cells is due to its replacement of oxygen.

CONCLUSION

The experiments here described appear to find their simplest explanation on the basis of the relation between the redox potentials and the rate of oxygen consumption of the cells. Development of the larvae falls into this picture. The effects of carbon monoxide seem to result only from the practical exclusion of oxygen. The converse action of cyanide and methylene blue supports this concept: cyanide stops oxygen use most effectively when acting on the stage of eggs or larvae least accelerated by methylene blue (the gastrula) and least effectively when acting on the stage (unfertilized eggs) most strongly accelerated by methylene blue. This relation is borne out in detail in all stages; and is supported by minor observation.

Changes in redox potentials within the eggs are considered to be established by the presence of reduced and oxidized metabolites, and reduced and oxidized links in the enzyme chain of oxidation. All of these redox participants must be affected by the introduction of redox agents like methylene blue and cyanide which act through their poising action. Their effects must lead toward or away from an optimum E_h in which the oxidizing enzyme chain acts most rapidly.

The interrelationship between the oxidation chain and the prevalent E_h values is a master key in understanding the changes in rate of oxygen consumption during development of echinoderm eggs, normally and under the influence of reagents. This concept is of general applicability.

SUMMARY

The oxygen consumption of eggs and larvae of *Asterias forbesii* and *Arbacia punctulata* was measured by the Warburg-Barcroft technique, in sea water, in sea water solutions of KCN (5×10^{-4} M) and methylene blue (0.002 per cent) and with atmospheres of carbon monoxide (99.5 per cent pure). Eight stages were studied: unfertilized and fertilized eggs, first cleavages, morula, blastula, early and late gastrula, and pluteus. Subsequent development after a period of two to three hours exposure to these reagents was followed in sea water.

Methylene blue increased oxygen consumption most when acting on unfertilized eggs, did not increase it for gastrula, and increased it slightly in the other stages. When transferred to sea water, the effects of methylene blue persisted in increasing the rate of development of larvae, prolonged their life and produced larger plutei.

Cyanide decreased oxygen consumption most strongly when acting on gastrulae, less so for other stages, and had little or no effect on unfertilized eggs.

These two agents have a converse action. This was shown by the antagonism between these two, as shown in this paper by the promotion of motility in cyanide paralyzed forms.

Carbon monoxide prevented gas consumption, subsequent fertilization and produced cytolysis. Methylene blue promoted the subsequent fertilization of CO-treated eggs.

These results are most simply accounted for on the assumption that the redox potential changes to a more positive level during progress from the egg to the gastrula stage and thereafter drops slightly. Methylene blue raises and cyanide depresses the positive redox potential. Carbon monoxide, as used in these experiments, indirectly depresses the redox potential by preventing the oxidized forms of the enzymes to exist. These effects take place in an oxidative enzyme chain whose members undergo reduction or oxidation. The whole system suffers changes under the named reagents, leading towards or away from the optimum E_h levels and maximum oxygen consumption.

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DEVELOPMENT OF THE PRIMARY GONADS AND DIFFERENTIATION OF SEXUALITY IN TEREDO NAVALIS AND OTHER PELECYPOD MOLLUSKS

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In connection with studies on the sexual differentiation and changes of sex in several genera of bivalve mollusks the writer has observed certain morphological characteristics of the gonads which seem not to have been reported previously. These concern particularly the types of cells which compose the gonads in the early stages of development and the provisions for the nourishment of the forming gametes. They pertain also to the earliest sexual differentiation of the primary gonia and their transformation into the functional gametes of the primary sexual phase. Some confusion exists in the literature because of failure to interpret correctly the significance of the two types of sexual cells which in young individuals of *Teredo navalis* and other ambisexual mollusks characterize this initial phase of functional sexuality.

ORIGIN AND EARLY DEVELOPMENT OF GONAD

In all the pelecypods examined the gonads originate from a group of cells situated in the posterior portion of the body, near the visceral ganglion and on the ventral side of the pericardium. As these primordial germ cells multiply they become more or less completely separated into two groups, situated symmetrically on the two sides of the body. By the continued multiplication of their constituent cells each group grows anteriorly in the surrounding mesenchyme or vesicular connective tissue to form the branching system of tubular follicles which characterizes the gonads of the pelecypods (Figure 1).

Two rather distinct types of gonads are found in these mollusks, each type being characteristic of certain genera. In Teredo, Bankia, Mya, Petricola, Barnea and some other members of the order Teleodesmacea the cells of the gonad soon become differentiated into large, vacuolated follicle cells and primary gonia. The follicle cells function as accessory nutritive cells. They occupy most of the space within the tubular follicle, the primary gonia being scattered along the periphery or near the central axis (Figures 1 to 4). In Ostrea, Pecten, Mytilus, Volsella and some other Prionodesmacea, on the contrary, the gonad is composed almost entirely of gonia, with only minute follicle cells (Figures 5, 6). In these forms the gametogenic cells obtain their nourishment directly from the surrounding vesicular connective tissue. An intermediate condition is found in Anomia and some other genera in which the gonadal follicles are associated with

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[1] Contribution from Scripps Institution of Oceanography, New Series No. 193.

vesicular nutritive cells, often arranged in follicles (Figure 7), or they may be composed of follicles with relatively few accessory nutritive cells.

The genital ducts of the adult open into the mantle cavity near the site of the original gonadal primordium or, in some species, into the kidneys and thence to the mantle cavity. When the individual has become sexually mature the branching gonadal follicles may extend throughout the mesosoma and, in the mussels and a few other pelecypods, even into the mantle beneath one or both valves of the shell.

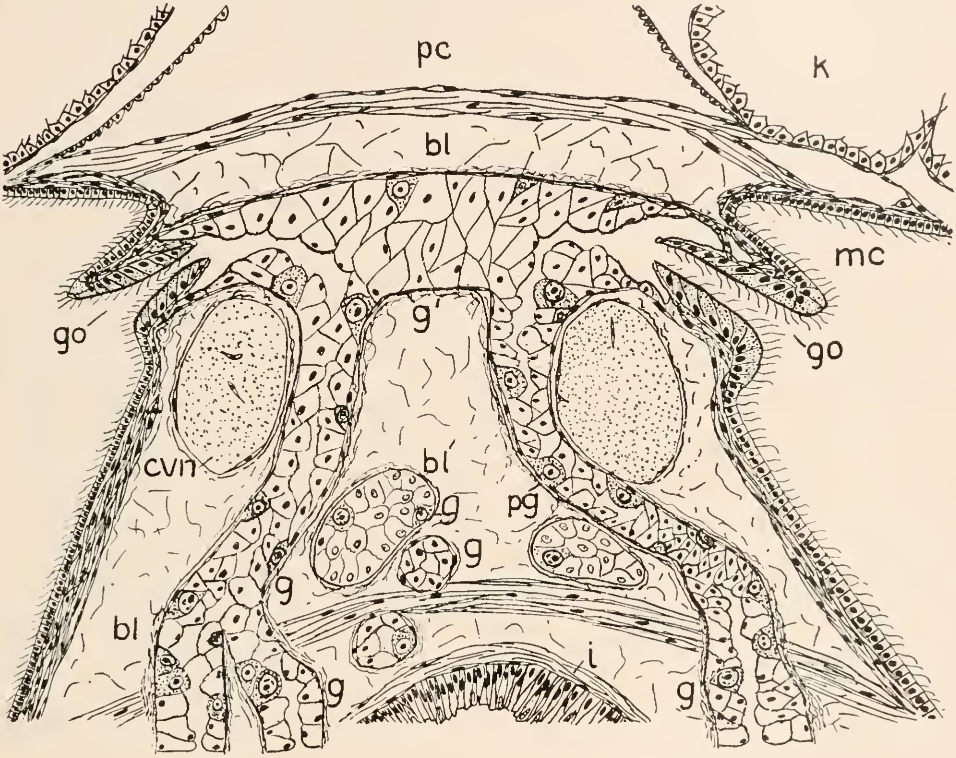


FIGURE 1. Portion of section through posterior region of body of young *Mya arenaria*, showing early stage in development of the tubular, branching gonads (*g*) composed of large, vacuolated follicle cells with primary gonia (*pg*) scattered along the periphery; *bl*, vesicular connective tissue; *cvn*, cerebro-visceral nerve cords; *go*, genital openings; *k*, kidney; *i*, intestine; *mc*, epibranchial chamber; *pc*, pericardial cavity. (Modified from Coe and Turner, 1938.)

SEXUAL DIFFERENTIATION

In *Teredo navalis*, which normally experiences alternating male and female phases of functional sexuality, the gonadal primordia consist of groups of cells, all of which are identical in appearance. But as soon as the primordia begin to branch out to form the tubular follicles, as described in a preceding paragraph, two distinct types of cells become distinguishable. At the end of each growing follicle there is a cap of undifferentiated nuclei in a continuous mass of cytoplasm (Figures 2, 4). As growth proceeds some of these nuclei become ecnlosed in large, vacuolated cells which function thereafter as accessory nutritive cells. A smaller number retain a larger portion of the terminal cytoplasm and become the primary gonia. As the follicle increases in length these primary gonia are left behind on the periphery of the follicle cells which otherwise fill the entire follicle (Figures 2, *A*, 4). The primary gonia multiply slowly as the follicles increase in length.

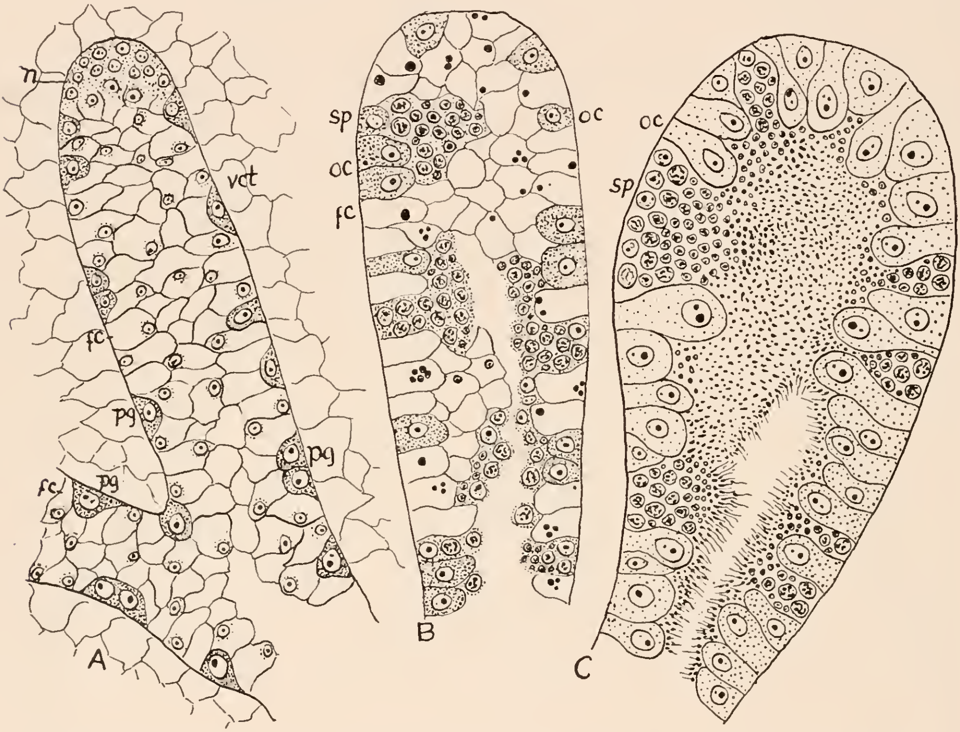


FIGURE 2. Development of primary gonad in *Teredo navalis*. *A*, sexually undifferentiated branching follicle, composed of large, vacuolated follicle cells (*fc*) with primary gonia (*pg*) scattered on the periphery; *n*, terminal cap of undifferentiated proliferating nuclei; *vct*, vesicular connective tissue. *B*, later stage with differentiated ovocytes (*oc*) and spermatogenic cells (*sp*) derived from the primary gonia; *fc*, remaining follicle cells with disintegrating nuclei and products of abnormal spermatogenic cells. *C*, follicle of primary male phase, with spermatogenic cells (*sp*) and spermatozoa filling the lumen and large ovocytes (*oc*) on the periphery.

No sexual differentiation can usually be detected in the primary gonia until the young teredo has attained a length of about 10 mm. In the warm season of the year, when the water is above 18 degrees C., this length may be reached within three to four weeks after entering the wood. Dwarfed individuals become sexually differentiated at a smaller size but at approximately the same age.

The spermatogonia are recognized as soon as the primary gonia begin proliferation into groups of nuclei within a cytoplasmic syncytium. These soon form primary spermatocytes with characteristic phases of synapsis (Figure 2, *B*).

Approximately half the total number of primary gonia become activated in this manner. An equal number differentiate into ovogonia and their resulting ovocytes.

Each of the rapidly growing ovocytes remains attached to the wall of the follicle until fully mature. The spermatogenic cells, on the contrary, multiply rapidly and soon fill the axis of the follicle (Figure 2, *B*). The follicle cells have meanwhile begun to undergo cytolysis and disintegration. Their nuclei first become pycnotic and are later cytolyzed. The collapse of the follicle cells forms a central lumen in the follicle and in this cavity spermatogenesis is completed (Figure 2, *C*).

With the continued growth of the ovocytes and a vast increase in the number of spermatogenic cells the follicles become greatly distended and the former lumen packed with spermatozoa (Figure 2, *C*). This is a typical hermaphroditic gonad but since the spermatozoa will usually become ripe and will be discharged before the ova are fully mature the individual is properly recognized as being in the primary, or protandric, or ambisexual male phase.

If all individuals experienced the same aspects of sexual differentiation there could be no difference of opinion as to their status, but an examination of the gonads of more than 3000 individuals shows wide variations in the proportion of spermatogenic and ovogenic cells present. This is true not only for different individuals but for different follicles of the same individual (Coe, 1933, 1934a). Photographs of some of these variations are shown by Coe (1933).

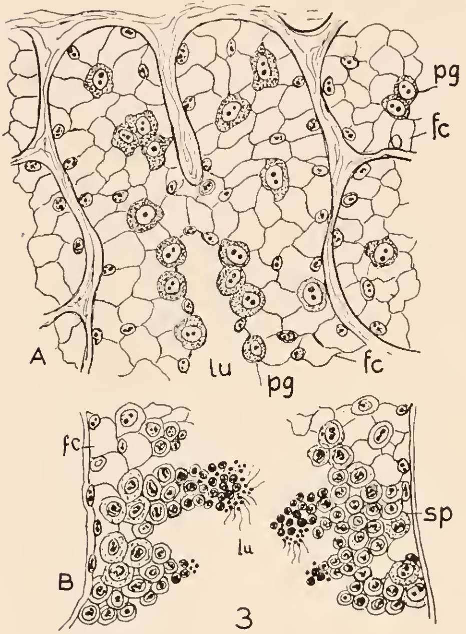
At one extreme are the so-called true males, in which the ovogonia, if present, fail to produce ovocytes. At the other extreme there are proterogynic individuals which form ovocytes exclusively in their first functional sexual phase, the usual primary male phase being aborted or inhibited (Coe, 1935). In exceptional individuals both spermatogonia and ovogonia are activated simultaneously, producing functional hermaphrodites. Some of these are capable of self-fertilization (Coe, 1941).

Both the true males and the proterogynic individuals must be considered as exceptions to the great majority of the population in which each individual normally functions first as male and later as female. The female phase will be followed by a second series of male and female phases if the life of the individual be sufficiently prolonged. This alternation or rhythm of sexual phases may be considered as characteristic of the species, although the mortality is usually very high before even two of the phases have been completed (Coe, 1934a).

Furthermore some of the typically protandric individuals approach more or less closely the true males in having relatively few ovocytes in the gonads. They retain the male phase longer than others which bear a closer resemblance to the proterogynic individuals in the appearance of the primary gonads (Coe, 1934a, 1936).

Because of this variability in the expression of ambisexuality, as well as associated differences in the time necessary to complete any one of the sexual phases, the proportion of individuals in each phase found in any sample of the population would seem to have less significance than has sometimes been assumed (Grave and Smith, 1936; Coe, 1936). It is obvious that if in such a sample twice as many individuals are found in one sexual phase as in another it may be merely indicative that the one phase lasts twice as long as the other. It might also indicate that some individuals are older than others, since the sexual phases appear in sequence during the life of the individual, with the exceptions already mentioned.

During the summer months there will ordinarily be fully three to five times as many individuals in the primary male phase as in the female phase at the first spawning period. This may occur as early as six weeks after metamorphosis. Three or four weeks later, after the change of sexual phase, the same individuals may be expected to show an approximation to a reversed proportion of male and female phases. There will be many deviations, however, since, as already mentioned, the primary sexual phase will be more prolonged in some individuals than in others and some will change quickly to the third (ordinarily male) phase. In the meantime younger members of the population will be functioning in the primary male phase.



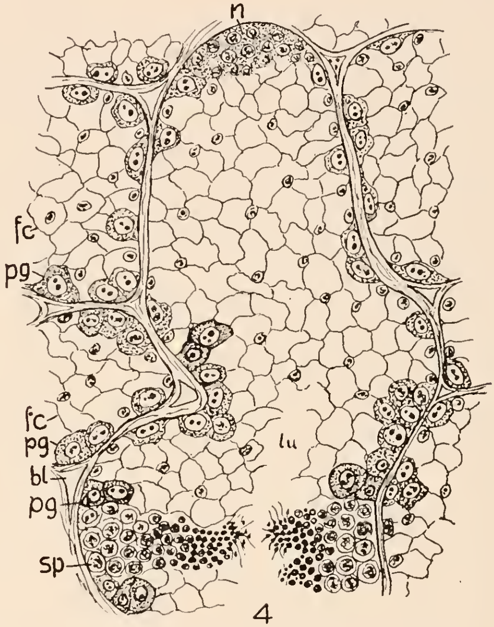
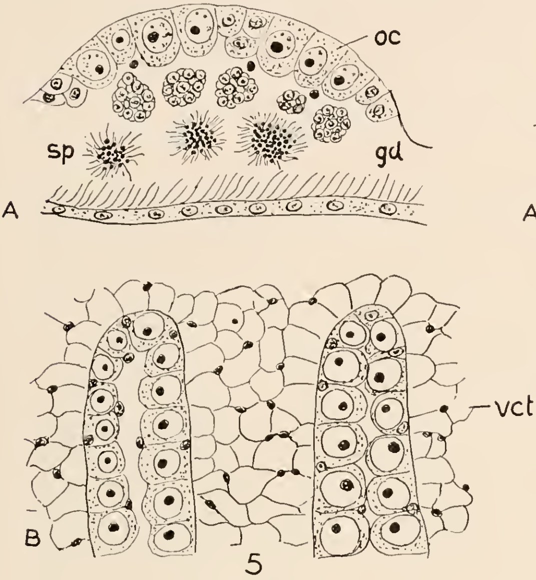


FIGURE 3. Portions of primary gonads of *Petricola pholadiformis*. *A*, sexually undifferentiated stage; follicle composed mainly of vacuolated, nutritive follicle cells (*fc*) with primary gonia (*pg*) along the central axis or lumen (*lu*). *B*, later stage in young male; most of the follicle cells have been cytolyzed to supply nourishment for the proliferating spermatogenic cells (*sp*).

FIGURE 4. Development of primary gonad in *Mya arenaria;* central follicle with terminal cap of undifferentiated nuclei (*n*), from which both follicle cells (*fc*) and primary gonia (*pg*) originate; lower portion of follicle shows later stage in which the follicle cells are being replaced by spermatogenic cells (*sp*), with spermatozoa adjacent to the lumen (*lu*). Note blood vessels (*bl*) and small amount of connective tissue between follicles.

The proportion of individuals in which the first sexual phase is female is thought by Grave (1942) to be larger than formerly reported (Coe, 1936) but the data presented do not seem to justify such a conclusion.

The following scheme, modified from Coe (1935), indicates such variations in the sequence of sexual phases as are inferred from a study of more than 3000 individuals at various ages. It is impossible to follow these phases in any single individual because the teredo dies soon after it is removed from the wood for examination. The presence of larvae in the gill chambers, however, is sufficient proof that the preceding sexual phase of the gonad was female.



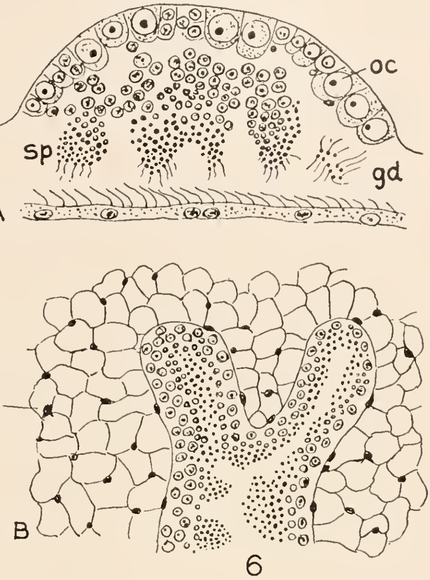


FIGURE 5. *Ostrea lurida*. *A*, portion of primary gonad showing typical ambisexual male-phase condition, with peripheral layer of ovocytes (*oc*) and developing sperm balls (*sp*) in the lumen; *gd*, ciliated genital duct. *B*, terminal portions of two follicles of secondary gonad in early female phase surrounded by much vesicular connective tissue (*vct*).

FIGURE 6. *Ostrea virginica*. *A*, portion of primary gonad in ambisexual condition. *B*, terminal portion of follicle surrounded by much vesicular connective tissue in early male phase of adult; letters as in Figure 5.

1. True male phase (exceptional) . . . Second male phase . . .
2. Ambisexual male phase . . . First female phase . . . Second male phase . . . Second female phase (if life be sufficiently prolonged).
3. Functional hermaphroditic phase (exceptional) . . . Male or female phase . . .
4. Female phase (exceptional) . . . First male phase . . . Second female phase . . .
5. Female phase (exceptional) . . . Second female phase (?) . . .

Many other pelecypods resemble the teredos in having the primary gonads composed largely of nutritive follicle cells. In some of these, of which *Petricola pholadiformis* may be taken as an example, the primary gonia are scattered along the central axis of the follicle (Figure 3). In others, as *Mya arenaria* (Coe and Turner, 1938), they are distributed along the periphery (Figure 4). In both cases the follicle cells are cytolyzed during gametogenesis. With the exception of an occasional hermaphrodite, all individuals of both species are strictly unisexual and the two sexes are approximately equal in number.

A different type of gonad is found in both the larviparous and oviparous oysters (Ostrea), in scallops (Pecten), mussels (Mytilus, Volsella), and numerous other bivalves. In these the gonads are composed almost entirely of gametogenic cells which receive their nourishment directly from the sourrounding vesicular connective tissue. The follicle cells are few and small (Figures 5, 6). As the gonadal follicles increase in size the surrounding nutritive connective tissue is utilized.

The larviparous oysters, of which *Ostrea lurida* may serve as an example, exhibit changes in sexuality during life, the sequence of sexual phases being similar to those mentioned for *Teredo navalis* (Coe, 1934). In the oviparous oysters, as *O. virginica*, there is likewise a strong tendency toward protandry, since about 70 per cent of the young individuals first function as males (Coe, 1938). The primary gonad usually contains antecedent cells of both sexual types (Figure 6, *A*). Functional hermaphroditism occurs occasionally and a few individuals change from the male to the female phase during their first spawning season. Thereafter the individual functions in one sexual phase or the other during all of each spawning season, but not infrequently the sexual phase changes between two spawning seasons (Burkenroad, 1937; Galtsoff, 1938; Coe, 1938; Loosanoff, 1942). In

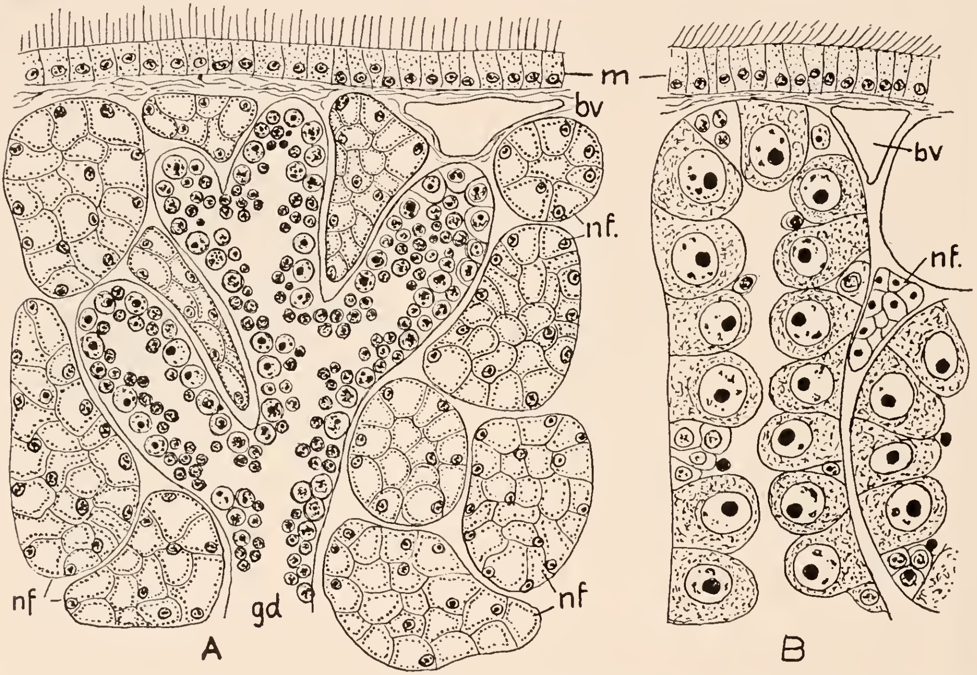


FIGURE 7. *Anomia simplex. A*, portion of primary gonad surrounded by nutritive tissues (*nf*). *B*, follicles of mature ovary in which the nutritive tissues (*nf*) have been almost assimilated; *bv*, blood vessels; *m*, mantle; *gd*, genital duct.

Venus the sexes are separate, following a juvenile, usually protandric, sexual phase (Loosanoff, 1937).

Gonads in some respects intermediate between the other two are found in Anomia and in some other genera. Here the gonadal follicles, which are composed almost entirely of gametogenic cells, are surrounded by nutritive tissues of similar configuration (Figure 7). As in the other types of gonads the nutritive tissues are utilized during the course of gametogenesis (Figure 7, *B*).

Summary

Two more or less distinct types of primary gonads are found in bivalve mollusks. The simplest type occurs in several families of the order Prionodesmacea, where the profusely branching follicles on each side of the body are composed mainly of gametogenic cells and each follicle is nourished by the surrounding mesenchyme or vesicular connective tissue.

In the second type, found mainly in the order Teleodesmacea, the branching follicles of the primary gonads are composed principally of large, vacuolated follicle cells of a nutritive nature, the primary gonia being scattered along the central axis or on the periphery. These nutritive cells are cytolyzed during gametogenesis.

In some bivalves intermediate conditions are found, with associated gametogenetic follicles and nutritive tissues.

In the ambisexual or hermaphroditic species, as *Teredo navalis*, the primary gonia are early differentiated into the two sexual types. Of these the spermatogonia usually, but not invariably, proliferate and complete gametogenesis in advance of the ovogonia, giving each follicle the appearance of a spermary with a layer of ovocytes on the periphery. As a general rule the ovocytes do not become fully mature until after the discharge of the spermatozoa. There are many variations in the relative proportion and time of spawning of the two types of gametes, however, and in exceptional cases the gonad is almost exclusively of the male or of the female type. Most individuals function first in the male phase, then change to the female phase, while some individuals experience in addition a second sequence of male and female phases. Less frequently a primary female phase precedes the first male phase. Individual differences in the combinations of the modifying hereditary sex factors are presumably responsible for most of these variations.

Each species studied, except those that are strictly unisexual, shows some variations in the sequence of male and female or functionally hermaphroditic phases.

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AN IMPROVED METHOD OF ASSAYING MELANIN IN FISHES[1]

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Some five years ago, the authors described a method of evaluating the melanin content of fishes (Gillichthys) kept under various experimental conditions, along with some preliminary results obtained by the use of this method (Sumner and Doudoroff, 1937). In the following year we published a brief account of similar experiments, conducted upon another teleost, *Gambusia affinis* (Sumner and Doudoroff, 1938). In these last it was found advisable to revise our earlier procedure in important ways.

Despite certain obvious shortcomings in the technique employed in those earlier studies, interesting quantitative relations were shown to obtain between the melanin extracted from our fishes and the optic stimuli to which they had been subjected. Whatever the precise nature of these relations, there could be no reasonable doubt that fishes (of at least two species) which had lived for some weeks upon black or dark gray backgrounds had a much higher melanin content than fishes kept upon various paler backgrounds. This was in full agreement with experiments involving the counting of melanophores, through which it had been shown that both the number of these cells per unit area of skin and the amount of melanin per cell may be greatly influenced by the background.[2] Much remained to be learned, of course, regarding the details of these relations.

In quantitative studies of melanin, gravimetric determinations seem to be ruled out by the impossibility of isolating this substance without sacrificing a large proportion of it during the process of isolation. Various methods of obtaining pure melanin have been developed by chemists whose object has been to purify it for chemical analysis, rather than to measure accurately the amount present in a given sample of tissue (e.g., Gortner, 1910, 1911; Salkowski, 1920; Heinlein, 1924).

Colorimetric methods were consequently resorted to early in our endeavors to assay melanin. We know of only two previous workers (Kudo, 1922; Vilter, 1931) who had attempted the measurement of changes of melanin resulting from experimental optical conditions, at least in fishes and amphibia. Recently, however, Dawes (1941) has published the results of some interesting experiments upon *Rana temporaria*.[3] His findings, so far as the effects of black and white backgrounds are concerned, fully confirm our own. Considering the facts just stated, however, it is obvious that Dawes is over-enthusiastic in his claim that his work "affords conclusive evidence for the first time that prolonged exposure of one of the lower vertebrates to . . . black background results in a marked increase in the melanin content of the skin."

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[1] Contributions from the Scripps Institution of Oceanography, New Series, No. 194.

[2] The work of ourselves and others in this field has been summarized by Sumner (1940b).

[3] Dawes's technique involved peptic digestion of the frogs' skins.

The employment of colorimetric methods in "melaninometry" is rendered possible by the solubility of this substance in dilute alkali.[4] Admittedly these methods involve some rather serious difficulties. Some of the primary requirements in the preparation of such a melanin solution are: (1) to retain without loss and unaltered the melanin contained in each sample of material; (2) to eliminate all cloudiness and obtain a perfectly clear solution; (3) to eliminate any colored materials other than melanin; (4) to avoid the production of pseudomelanins ("melanoidins") which sometimes result from the action of strong acids upon proteins.

The procedure outlined below is the outcome of experimentation by the authors, which was commenced in 1935. While it is empirical in many details, we believe that the various steps are theoretically justifiable. Many of these steps are based upon the methods of previous workers, but the combination is ours.

Comparison with our earlier report (1937) shows that somewhat extensive changes have been made in this procedure. Since little melanin is contained in the more massive tissues of the body (muscles and viscera), the presence of which increases the difficulty of preparing clear solutions, it was decided to exclude these tissues; hence paragraphs 4, 5, and 6 of our revised procedure (cf. Sumner and Fox, 1935).[5] Again, peptic digestion, followed by centrifuging, was replaced in our revised technique by boiling in 6 per cent HCl, followed by dialyzing (paragraphs 22 to 25).

Our most recent procedure follows:

(1) The fishes (unfed for at least two days) were killed in chloroform vapor; (2) dried on paper towels; (3) each individual measured, and each lot (those combined to form a single sample) weighed; (4) dipped into boiling distilled water $\frac{3}{4}$ minute, then cooled in cold water; (5) the skin was removed from the entire body and head and placed (along with fins) in distilled water; (6) water warmed to 60° C. and beakers placed in oven at that temperature for one hour (to remove gelatin); (7) 95 per cent alcohol substituted for water, changed once, material bottled and kept in the dark, sometimes as long as five or six weeks; (8) alcohol poured off and material subjected to Soxhlet fat-extration (in 150 cc. thimbles) for three hours, using 250 cc. of alcohol-ether mixture (2 : 1); (9) material left in thimbles and dried in oven at 60° for several hours; (10) decalcified in one per cent HCl for one hour at 60° (5 cc. per gm. of original weight of fish, *not of skin*); (11) acid filtered off through no. 2 sintered-glass filter, residue washed with 500 cc. distilled water; (12) residue from filter hydrolyzed by boiling one hour in 0.2 per cent NaOH (5 cc. per gm. of original weight of fish) in three-liter flask, under reflux condenser; (13) resulting mixture filtered through no. 2 glass filter, to remove undissolved impurities; (14) filtrate, when of sufficient volume, divided into two parts ("a" and "b") to be run separately as checks; (15) HCl added, drop by drop, to each lot, until neutral point is just past and a precipitate forms which includes the melanin (important that approximately the same pH

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[4] Strictly speaking, these are not molecular solutions, but very fine colloidal suspensions. They may be very clear, however, and there is little or no Tyndall-effect unless the suspensions are highly concentrated.

[5] As we were primarily interested in changes in the external pigmentation (that of the skin), we had no object in retaining the considerable amount of melanin contained in the peritoneum or in the eyes.

is reached for all samples); (16) material (liquid and precipitate) transferred to centrifuge tubes (approximately 100 cc. capacity), centrifuged 15 minutes at approximately 1760 r.p.m., liquid[6] poured off (precipitate adheres closely to bottom of tube); if all material from one sample is not, at first, contained in a single tube, it is combined into one tube and re-centrifuged; (17) 95 per cent alcohol carefully poured onto precipitate in tubes (4 cc. per gm. of original weight of fish), left at least ½ hour; (18) this alcohol removed by suction (using a glass tube, finely drawn out), and replaced by same amount of absolute alcohol, left at least one hour; (19) alcohol again removed and ether (the same quantity) substituted, precipitate thoroughly stirred, left at least one hour; (20) materials centrifuged ½ hour, and ether removed by suction; (21) dried in oven at 60°; (22) dissolved (while still in centrifuge tubes) in 0.2 per cent NaOH (1 cc. per gram of original weight of fish); (23) equal volume of 12 per cent HCl added, making a concentration of (nearly) 6 per cent acid; (24) transferred to flasks and boiled one hour under reflux condensers; (25) liquid, with precipitate, transferred to celloidin ("Parloidin") dialyzers, suspended in one-liter beakers of distilled water. Left at least three hours, during which water is changed five times; (26) material poured (and rinsed) from each dialyzer into graduated cylinder, enough 5 per cent NaOH added to make a 0.2 per cent solution, when liquid has been brought to final required volume (3 cc. per gm. of original weight of fish); (27) solutions returned to flasks and boiled for one hour under condensers (if necessary the condensers are left off for a time and liquid evaporated sufficiently to bring the volume down to *somewhat less* than the required final volume, to allow for rinsing); (28) solutions returned to graduates and enough distilled water added to bring them as nearly as possible to required volume (3 cc. per gram of fish); (29) passed through no. 2 sintered-glass filters, previously dried in oven (no material loss of melanin by adsorption is found to take place in a filter of this grade of porosity).

The melanin "solutions" are now satisfactorily clear and ready for direct colorimetric reading by transmitted light. This is obviously more satisfactory than our earlier, more involved procedure (1937).

The colorimeter employed in these studies (the Ives Tint-Photometer) has been employed by the senior author and collaborators for a number of years in several lines of investigation. Since it has already been described rather fully (Sumner and Fox, 1933, and earlier), no detailed account is called for. One beam of light, reflected from a block of white magnesium carbonate, is passed through an absorption-cell containing the solution under examination, another beam through a neighboring cell containing distilled water. An adjustable diaphragm controls the amount of light emerging from the latter cell so that the intensity of the beams emerging from the two cells can be brought to equality. Before reaching the eyes of the observer however, both beams are passed through one of a series of color filters. Using each of these filters in turn, the two halves of the field are balanced and the readings recorded. For the present studies, three filters have been employed: red, green, and blue.[7] It is evident that in the use of this instrument high readings represent low melanin values and vice-versa. The highest (100 per cent) readings are obtained from pure water.

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[6] This liquid is ordinarily clear and of a pale straw-color, quite different from that of a diluted melanin solution. Solutions from "black" fishes (i.e., ones from black background) were found, however, to be about 80 per cent more deeply tinted than ones from "white" fishes when several pairs of samples were compared. At most, the coloration is feeble.

These successive readings constitute the primary data upon which our assays of melanin are based. Each observer repeats his readings several times. When two observers work in collaboration, their readings are averaged.[8]

For the interpretation of these figures for color-values obtained with the tint-photometer it is necessary to prepare a set of curves based upon melanin solutions of known concentration. Through the kindness of the late Professor R. A. Gortner, of the University of Minnesota, we obtained a sufficient quantity of purified melanin, prepared by him from the skin and other tissues of the "silky" fowl. Curves based upon tint-photometer readings of a series of dilutions of a solution (.0667 gm. per 100 cc. 0.2 per cent NaOH) are shown in Figure 1. These are the curves which have been employed by the senior author in deriving the values reported for Girella and Fundulus in this issue (pp. 195–205). The

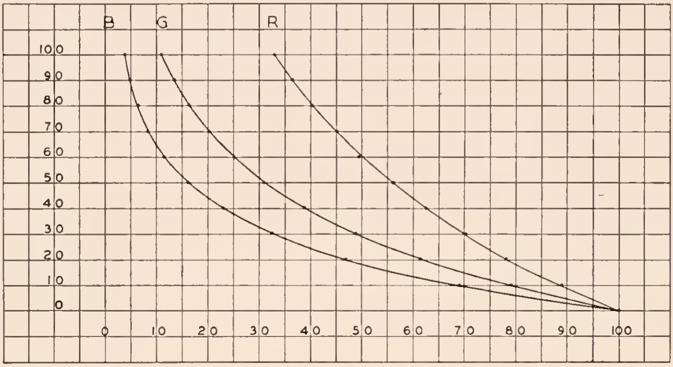

FIGURE 1. Tint-photometer readings of melanin solutions of various concentrations. The highest concentration ("100%") was prepared by boiling dried melanin for three hours in 0.2 per cent NaOH solution in the proportion of 0.667 gms. to 100 cc. This was variously diluted with NaOH solution. Abscissas = tint-photometer readings; ordinates = dilutions (expressed as percentages of "100%" solution).

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[7] Specifications for these filters, taken by means of the Bausch & Lomb Visual Spectrophotometer are as follows:

		mμ
Red	Maximum transmission (70% ±)	760–650
	Lower limit (1% transmission)	602
Green	Maximum (18% ±)	530–520
	Upper limit (1%)	610
	Lower limit (1%)	475
	Points of half transmission	500, 565
Blue	Maximum (31% ±)	460–450
	Upper limit (1%)	515

(The blue has been referred to as "blue-violet" in our earlier publications.)

[8] There was, not unexpectedly, a personal equation in these readings. In the case of the last ten samples studied by us, melanin values based upon the readings of the senior author were 1.7 per cent higher than those obtained by the junior author. The mean differences, regardless of sign, averaged 2.0 per cent.

melanin values therein presented are expressed in milligrams per gram of the original weight of the fish.

Allowance must be made for the probability that melanins derived from different organisms are not all chemically identical. Hence, these figures for fish melanin, based upon comparisons with the melanin of a bird, may be no more than rough approximations. In our present studies, however, we are chiefly concerned with relative rather than absolute values, our main object being the discovery of relations among our various experimental groups.

We feel justified in basing our melanin titer upon the weight of the fishes, rather than upon the surface area of the skins, for the reason that the skin of an animal has a third dimension, thickness, which probably varies roughly in proportion to its other dimensions. (It is obvious that the wet weight of the scraped-off skins would be of no significance for this purpose.) It is of interest, however, that when the melanin content, *per square of body length*, is computed (in the absence of any actual measurements of surface areas), the essential relations among the various experimental lots remain unchanged.

Previous writers have called attention to the production of dark "melanoid" substances through the action of strong acids upon proteins. These would obviously interfere with colorimetric measurements of melanin. That boiling with 6 per cent HCl can have had no such effect upon our material seems to be proved by a special test which was made for this purpose. Twelve goldfishes, very nearly devoid of melanin in their skins, were subjected to our standard procedure in three lots of four fishes each. These agreed in yielding solutions hardly tinted enough to permit of readings with the tint-photometer. The slight coloration was about 2 per cent of that of the "100 per cent" standard melanin solution (Figure 1). Moreover, in six cases, one to two additional hours of boiling in HCl (step 24, p. 189) resulted in a slight mean *loss*, rather than increase in the melanin titer, though this difference was of doubtful significance.

The same cannot be said, however, of extending the time of boiling in sodium hydroxide. Six samples which were boiled for three hours gave melanin values averaging 7.4 per cent lower than portions of the same solutions which were boiled the usual one hour.

Furthermore, as we have reported earlier (Sumner and Doudoroff, 1938) alkaline solutions of melanin invariably deteriorate (become paler) at ordinary room temperatures, even when kept in the dark. Thus a set of three samples from black-adapted fishes underwent a mean apparent loss in melanin content of 9 per cent in 24 hours, 18.6 per cent in five days, and 24.3 per cent in 12 days. These figures are based upon the means for the three color filters. Actually, there were wide differences between the figures for loss, when based upon readings with the different filters. Thus, at the end of 12 days, the figures seem to show a decrease of 33 per cent, if readings with the red filter alone are considered; only 16 per cent if the blue ones alone are considered. This obviously implies a change in the color as well as in the density of our solutions.

Readings taken of our "standard" melanin solutions at the end of 12 days presented much the same picture. In the "70 per cent" solution[9] (corresponding approximately to that derived from black-adapted Girella) the seeming loss in melanin concentration, when based upon the mean of readings with the three filters, was 20.5, a figure not far from that shown by the solutions of fish melanin. Here, too, the separate figures, based upon the three filters, ranged from 27 per cent (red) to 15.7 per cent (blue).

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[9] Meaning 70 per cent of the concentration of the strongest solution (taken as 100 per cent) upon which Figure 1 is based.

Some further optical properties of a melanin solution, both when freshly prepared and after standing in the dark for 12 days, are portrayed in Figure 2. This is based upon readings with a Bausch and Lomb Visual Spectrophotometer of a solution having the same concentration as the "100 per cent" solution of Figure 1. The "curve" of transmission, at least for the freshly prepared solution, turns out to be a nearly straight line, with a practically uniform gradient from blue through red.

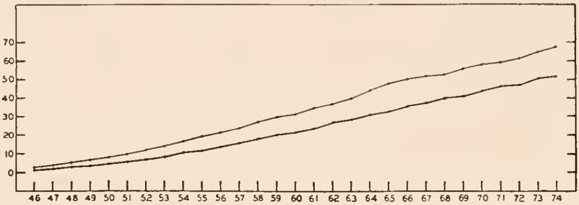

FIGURE 2. Curves of transmission of various wave-lengths by "100%" solution of purified melanin (see legend for Figure 1). Lower curve = freshly prepared solution. Upper curve = same solution after 12 days. Abscissas = wave-lengths at 10-mμ intervals; ordinates = percentages of transmission in comparison with distilled water.

When a fresh "70 per cent" solution is compared with a 12-day-old solution of the same original strength, we find an increase of 22 per cent in the reading with the red filter, an increase of 41 per cent in the reading with the blue. This might be construed as an increase in transmission toward the blue end of the spectrum. It must be remembered, however, that with increasing dilution, the transmission of the various wave-lengths through a colored solution tends to equalize, and that the least transmitted wave-lengths (in this case blue) will increase much more rapidly than the more transmitted ones (in this case red). In passing from a "70 per cent" to a "51 per cent" melanin solution, for example, we find an increase of 22 per cent in the "red" reading, and an increase of 89 per cent in the "blue" reading. Thus the much smaller increase in the transmission of blue rays through the 12-day-old sample, in comparison with the diluted fresh sample, denotes a shift toward the red on the part of the former. The figures upon which Figure 2 is based likewise reveal a greater relative transmission of the red rays than would result from simple dilution.

This lability of melanin solutions makes it necessary to employ boiling periods of uniform length in preparing them, and to make the photometric readings as soon as possible after the solutions are prepared—three or four hours at most. The unavoidable losses are thus kept constant, and the figures for the various lots may be regarded as strictly comparable.

A chief reason for our dissatisfaction with our earlier procedures (1937, 1938) was the frequent appearance of considerable differences in the yield of samples from which identical results had been expected. So far as such unexpected differences related to different lots of fishes having the same experimental history, there was, however, no real ground for concern. Later studies of melanophore density (Sumner, 1940, *a* and *b*) have emphasized the enormous variability in this respect within each of the experimental lots. There was considerable overlapping, for example, between the frequency distributions of melanophore numbers in even the "black" and "white" series of Lebistes.

Of more serious import are differences in results obtained from portions of the same sample of material which, after division, have been subjected to identical treatment. Such discrepancies, which were earlier encountered to a discouraging degree, have been largely, though not wholly, eliminated in our later studies. Comparisons of figures for "a" and "b" portions of the same sample (step 14, above) give us something of an index of the degree of constancy of our results. In 34 cases in a recent study, in which readings from two such portions were obtained, the mean difference between these portions was 3.2 per cent. This figure, it is true, indicates only a moderate degree of precision, but it is small compared with most of the experimentally produced differences upon which our conclusions are based. In 28 of these 34 cases, the differences between our "a" and "b" sub-samples were under 5 per cent. In the remaining six, they were 5 per cent or more, the largest figure being 12.3 per cent. No explanation can be given for these exceptional cases. Fortunately, they seem to become less frequent with increasing experience.

The results of a rather extensive application of the technique here described are presented in another paper in this issue.

We make grateful acknowledgment of the kindness of the late Professor R. A. Gortner of the University of Minnesota in supplying us with samples of purified melanin, and for valuable information; to our colleague, Dr. D. L. Fox for the use of the spectrophotometer under his charge as well as for advice on various matters, and to Mr. Sheldon Crane for assistance in the photometric work.

Summary

A method is described of preparing a solution (transparent colloidal suspension) of the melanin of fishes, and of assying this colorimetrically. The method includes hydrolysis of the tissues in a boiling alkaline solution, precipitation, centrifuging, further hydrolysis in acid and removal of acid and digestion products by dialysis; finally dissolving the melanin in dilute NaOH. Readings of the various samples were made with a tint-photometer, but a spectrophotometer could have been employed, perhaps, to advantage.

For the interpretation of these readings, curves were drawn, based upon solutions of purified melanin of known concentration.

The transmission "curve" of the various wave-lengths through such a melanin solution was likewise obtained by the use of a spectrophotometer. This proved to be a nearly straight line from a low point in the blue to a high point in the red.

With lapse of time, alkaline "solutions" of melanin rather rapidly deteriorate, even in the dark, becoming increasingly transparent to all wave-lengths, but proportionately more to the red than would result from mere dilution.

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A FURTHER REPORT UPON THE EFFECTS OF THE VISUAL ENVIRONMENT ON THE MELANIN CONTENT OF FISHES[1]

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INTRODUCTION

That a sufficiently long sojourn upon a black or white background may result in a marked increase or decrease in the pigmentation of fishes and amphibia has been shown by several investigators (Cf. recent review by the present author, 1940*b*). Sumner and Wells (1933) found pronounced changes of this sort in *Lebistes reticulatus*, in which not only the number of melanophores per unit area of skin was found to be markedly affected, but also the amount of melanin per cell. These changes were portrayed unmistakably in our published photographs, but no attempt was made at that time to measure their extent.

For two other fishes, *Gillichthys mirabilis* and *Gambusia affinis*, Sumner and Doudoroff (1937, 1938) made quantitative determinations of the amount of melanin present in animals of different experimental history. These determinations were beset with considerable obstacles which seriously affected their precision. Certain conclusions were, however, established with reasonable certainty in both of these series of experiments. (1) Fishes of "black" history yielded considerably more melanin than fishes of "white" history, while those from intermediate backgrounds (grays) were intermediate in this respect. (2) Within broad limits, the intensity of the incident light was a minor factor in the production of such differences.

A more definite quantitative relation was indicated as possible between the albedo[2] of the background and the amount of melanin produced (or retained). In Figure 3 (Sumner and Doudoroff, 1937) and Figure 1 (Sumner and Doudoroff, 1938), it is evident that the relation between these two variables is not a linear one. If the figures for albedo stated on page 213 (1937) be converted into percentages of "white" (the latter being regarded as 100) and if 0.74 be taken as the value for "black," the Gillichthys values of Table I, "A" (means), form a nearly perfect logarithmic series.[3] This relation does not hold at all well for the "B" fishes, however, though the arrangement here is likewise of the "hollow curve" type.

The relations shown by the "A" set of these fishes suggested as possible the rule that the amount of pigment produced (or retained) varied inversely as the logarithm of the albedo of the background. The analogy between such a formulation and the so-called "Weber-Fechner Law" in human sense-physiology was at once recognized.

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[1] Contributions from the Scripps Institution of Oceanography, New Series, No. 195.

[2] By *albedo* is meant the specific reflectivity of a given surface, i.e., the proportion of incident light which that surface reflects.

[3] This mode of treating the data is not in the least arbitrary. Our original attempt to state the albedo of our "white" in absolute terms was not successful and has been abandoned. But the figures for "albedo," referred to "white" as a standard, are fairly exact. The figure of 0.74 for "black" is taken from Sumner (1940a). While only approximate, it is certainly a fairer figure than 0.

Because of the difficulties encountered in our earlier attempts to assay melanin, the present author next resorted to counting melanophores in definite areas of the skin of *Lebistes reticulatus* (Sumner, 1939, 1940*a*). Accurate counting was rendered possible by causing the pigment-masses of the melanophores ("melanosomes") to concentrate through the action of adrenalin.

In these experiments, the previously observed gradient in the effects of the various backgrounds, from black to white, was clearly manifest. And again, the logarithmic relation between stimulus and pigmentation was plainly indicated, though the values for the "black" fishes were somewhat too low (1940*a*, Figures 3 and 4). Once more, any differences due to the intensity of general illumination, if significant at all, were slight in comparison with those resulting from the shade of the background.

While there could be no doubt that the melanin content of black-adapted fishes was greater than that of white-adapted ones, it was plain that more exact, quantitative expressions for these differences in melanin content were desirable.

Further endeavors to perfect a method of assaying melanin were resumed by Sumner and Doudoroff, who have discussed the outcome of their completed task in this issue of the *Biological Bulletin* (pp. 187–194). The present paper reports the results of two series of experiments in which this method has been applied successively to two species of fish, *Fundulus parvipinnis* and *Girella nigricans*. Since, of these two, the results from the latter species were of far greater significance, they will receive the principal attention.

I must here acknowledge the valuable assistance of Dr. Peter Doudoroff and Mr. Carl I. Johnson in the preparation of some of the equipment here employed, and of Mr. Urless Lanham and Mrs. J. F. Wohnus in connection with the care of the fishes and some of the subsequent laboratory procedure. Acknowledgment is also due Messrs. P. S. Barnhart and C. W. Palmer for obtaining the supply of fishes.

METHODS

In the case of Girella, eight to ten of the fishes, averaging (two months later) about 60 mm. in length, were placed in each of the 24 experimental bowls. Running sea-water was supplied to these. The fishes were fed three times a week, chiefly with beach-worms (Thoracophelia) and canned shrimps. Debris (feces and food remains) was removed from the bottoms of the bowls by siphoning, nearly every day, and the bowls were scoured once a week to remove bacterial and algal growths.

The bowls were kept in six cabinets, painted white within and lighted by electric lamps overhead. Four bowls of the same albedo were kept in each of five cabinets, lighted by a 100-watt lamp. A second set of four black bowls was kept in the sixth cabinet, lighted by a 10-watt lamp. The cabinets were all kept lighted night and day.

In the case of Fundulus, only five cabinets were employed, all with 100-watt lights, and the extra set of black bowls was omitted.

The intensity of illumination in the more strongly lighted cabinets, as determined with a Weston Photronic Cell placed at the customary water-level in the bowls, the latter being covered by a $\frac{1}{4}$-inch screen, was about 33 foot-candles. In the less brightly lighted cabinet, it was about 2 foot-candles.

The bowls were of clear, uncolored glass, with nearly straight sides, 24 cm. in diameter and 15 to 17 cm. deep. As has been our practice, these bowls were painted on the outside with several coats of auto enamel. Black and white enamel were used, singly and in mixtures which provided the three shades of gray. In order to eliminate bright gleams, from surface reflection, the bowls were given a frosted surface by sand-blasting on the inside. This surface becomes transparent when covered with water.

Albedos were determined by a method described more fully earlier (Sumner, 1940*a*). Light reflected from the bottoms was measured by the use of a photronic cell and galvanometer. The readings thus obtained did not reveal the absolute albedos of the surfaces in question (i.e., ratios of reflected to incident light), since it was impracticable with available equipment to measure the incident light reaching the bottom of a bowl nearly filled with water. This was not necessary, however, because the important thing to know in these experiments was the relative reflectivity of these various surfaces, as compared with one another. As already stated, these figures were reduced for present purposes to a common standard, the reflectivity of the white bowls being taken as 100.

Since, as already indicated, the bottoms of the bowls were unavoidably befouled part of the time, another set of albedo readings was taken with the bowls in this state. For this purpose, an attempt was made to reproduce their average condition preceding the—usually daily—siphoning off of the debris.

TABLE I

Albedos of the various backgrounds, relative to white as a standard

	Without debris	With debris	Approximate average condition
White	100.00	100.00	100.00
Pale gray	38.17	37.31	37.74
Medium gray	17.84	17.77	17.80
Dark gray	6.56	7.22	6.90
Black	0.94	1.94	1.42

The result was necessarily only a rough approximation. Table I gives the figures for each type of bowl, with and without the debris. The figures in the third column, which represent an average of the clean and the dirty conditions of the bowls, are the ones here employed in considering the relations between albedo and pigmentation.[4]

The present table gives the albedos of the bowls used for Girella. The set used for Fundulus received the same shades of paint (many of the bowls were identical), with the exception of the "dark gray" bowls, which were unintentionally made considerably paler, having an albedo of 11.79, instead of 6.90.

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[4] In Table I of Sumner, 1940a, the figures in the column headed "With Debris" are the ones employed in the text discussions of that paper, but those figures are based upon a less extreme condition of fouling than that dealt with in the present paper.

In the Girella experiments, about half of the fishes were removed for the melanin assay two months (59 to 61 days) after the commencement of the experiment, the remainder being removed two months later (a total of 122 to 123 days). Four samples were taken from each type of background on each of these occasions. Each sample consisted of from three to five fishes. Two series of Fundulus were likewise assayed, after periods of 24 to 26 days and 57 to 59 days, respectively. Here the four samples from each background consisted of five to seven fishes.

The treatment to which this material was subjected has been described by Sumner and Doudoroff in the present number of the *Biological Bulletin* (pp. 187–194).

RESULTS

For Girella, notes were made on the appearance of the living fishes near the close of each period of the experiment.

(1) Differences of shade, resulting from the influence of the backgrounds, were very marked. Most of the fishes in the black bowls were so black as to be nearly invisible against these backgrounds. Those in the white bowls were very pale, appearing almost white (for a few seconds only) when transferred to a black bowl. Fishes in the intermediate bowls were correspondingly graded.

(2) Except in the black bowls, and to a less degree in the dark gray ones, however, the adaptation was far from complete, even after four months. The white-adapted fishes, while very pale, appeared far from white, *on their own backgrounds*, and could not even be regarded as well concealed. The fishes on pale and medium gray were also considerably darker than their respective backgrounds.

TABLE II

Melanin content (mg./gm.) of Girella killed after two months

Black (10W)	Black (100W)	Dark gray	Medium gray	Pale gray	White
1.35	[0.91]	0.86	0.78	0.63	0.81
1.40	1.37	0.95	0.82	0.68	0.82
1.60	1.52	1.03	0.93	0.80	0.89
1.61	1.55	1.21	—	—	0.93
1.49	1.48	1.01	0.84	0.70	0.86

TABLE III

Girella killed after four months

Black (10W)	Black (100W)	Dark gray	Medium gray	Pale gray	White
1.33	1.22	0.88	0.63	0.50	0.61
1.51	1.40	1.02	0.71	0.57	0.62
1.51	1.55	1.12	0.73	0.61	0.65
1.58	1.57	1.22	0.91	0.64	0.79
1.48	1.43	1.06	0.74	0.58	0.67

(3) Great individual differences of shade were sometimes manifested among fishes in the same bowl, particularly in the medium and dark gray bowls. Among the latter it was noted that the two extremes could even be characterized as "pale" and "very dark," relative to their background. Whether or not such visible differences resulted from actual differences in the amount of melanin was not determined. Apparently, they were more or less permanent for the individuals concerned.

(4) The greater part of the induced differences of shade, even after four months, was of the transitory ("physiological") type. Strikingly rapid changes were still visible, following the transfer of a fish from one background to another. Again, the effect of the chloroform vapor with which the fishes were killed was to level down these differences, the pale fishes becoming much darker and vice-versa. While no figures are available for the color differences of the living fishes, it is certain that the difference between those of "black" and "white" history was many times the maximum difference ($2\frac{1}{2}:1$) shown by the melanin extracted from these same lots.

Figures for the melanin content of the skins of the various lots of Girella are shown in Tables II and III,[5] and some of the relations among these values are depicted graphically in Figures 1 and 2. Each of the values comprised in the tables is derived from one of the "samples" of three to five fishes referred to above, and in most cases it represents the mean of two sub-samples, into which the dissolved material was early divided. The figures in each column are arranged in order of magnitude, without reference to the chronological order of the analyses.

The range of magnitude displayed in each of the columns of these tables is plainly considerable. This despite the fact that each of these figures represents the mean condition of several individuals. Considering the very great individual variability of some fishes in respect to pigmentation, however, these differences among the melanin values for small samples are not surprising (Sumner and Doudoroff, this issue, p. 192).

It seems probable that the figure in brackets in Table II (0.91) is due to some accident or error of procedure, though no such error was perceived at the time. This low value is not approached by any other of the 16 figures comprised in the four columns for "black" fishes in the two tables. It will also be noted that the mean for the column containing it (black, 100W.), when the aberrant figure is omitted, agrees rather closely with the other three means for "black." If this (possibly correct) figure is included, the mean for its column is 1.34.

The two vacant spaces in Table II result from the accidental loss of the corresponding samples of material.

Aside from one feature, the curves (Figure 1) show much the same form as those presented in previous papers (Sumner and Doudoroff, 1937, 1938; Sumner, 1940, *a* and *b*). From black to pale gray, the arrangement of the values approaches very closely a logarithmic one, at least for the 4-month series (Figure 2).

In one respect, however, the curves based upon these experiments differ markedly from any of those shown in our earlier papers. In the present case, there is an actual rise from pale gray to white, the reality of this relation being emphasized by the fact that it is shown in both the 2-month and the 4-month series. Here we have an abrupt departure from the logarithmic relation. The possible significance of this will be discussed later.

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[5] These melanin values were obtained by reference to curves based upon "standard" solutions of melanin derived from silky fowls (see preceding paper, p. 190). It can hardly be assumed that they represent with any exactness the absolute quantities of melanin in our fishes.

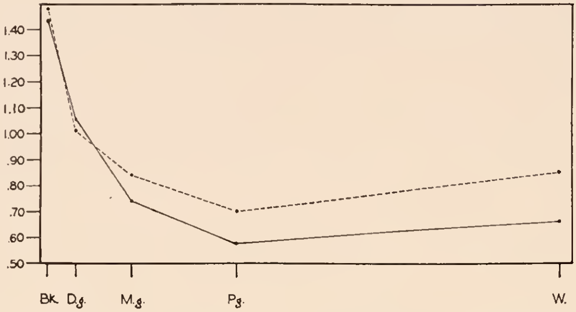

Figure 1. Melanin content (milligrams per gram of original weight of the fishes) of Girella, after sojourn on various backgrounds; broken line after two months, continuous line after four months. Abscissas = albedos; ordinates = melanin.

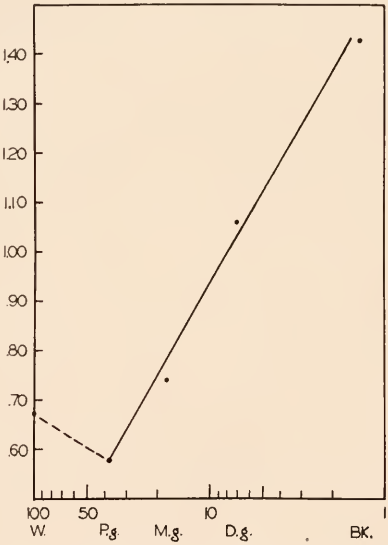

Figure 2. Melanin content of the 4-month series plotted against the logarithms of the albedos of the backgrounds.

Comparison of the 2-month and the 4-month series brings out certain points of interest. In respect to the values for black and dark gray, the two series differ but slightly from one another. Beyond the latter point, however, the two curves diverge steadily, that for the 4-month fishes falling well below that for the 2-month ones. This is not surprising when we consider that freshly caught fishes, when subjected to these artificial conditions, commonly lose pigment freely upon the paler backgrounds, but undergo little increase upon the darker ones.[6] This loss, as might have been expected, has progressed considerably further in four months than in two. It is not impossible, indeed, that longer subjection to the experimental conditions would have resulted in depressing the value for "white" below that for "pale gray."

To consider these differences quantitatively, we find that after two months the melanin yield of the "black" fishes was about 1¾ times that of the "white" ones, and more than twice as great as that of the "pale gray" ones. For the 4-month series, the figures are 2 + and 2½, respectively. As already stated, these differences are slight in comparison with the very great differences in the appearance of the living fishes.

Fundulus, as already stated, yielded far less instructive results than Girella. Earlier experiments had shown that visible responses to backgrounds were much less pronounced in the former than in the latter. Indeed, the living "black" fishes were far from black and did not seem to darken further after the first few days.

Mean melanin values (mg./gm.) for the 2-month period are as follows:

Black	0.37
Dark gray	0.40
Medium gray	0.36
Pale gray	0.34
White	0.26

These figures are based upon four samples (five to seven fishes each) in the case of the three grays, only three samples in the case of white and black. The significance of any of these differences may well be doubted, with the exception of that between "white" and the other four members of the series. In general, the variability among the figures for each background is high.

Comparing the figures for Fundulus with those for Girella, it is plain that the former, in all cases, is a much less highly pigmented fish than the latter. The ratio, indeed, is nearly 1 : 4. Again, the influence of the background is much less pronounced in Fundulus than in Girella. In the former it has been seen that there is little or no difference in melanin content between the fishes kept on black and those kept on the various shades of gray, while the "white" fishes seem to have lost only 30 per cent of their melanin.

Discussion

Despite the marked differences shown by the different fishes in our present and similar previous experiments, certain common features are obvious in their reactions to the background. In every case, among these various fishes (Gillichthys, Gambusia, Lebistes, Girella, Fundulus) quantitative changes have been brought about in the amount of melanin contained in the skin. In all of these, except Fundulus, the greatest melanin content was shown by the black-adapted fishes, and in all except Girella the lowest melanin content was shown by the white-adapted fishes. For the most part, too, the intermediate values for this pigment were graded in the order of decreasing albedo of the background. Exceptions to this were the position of "white" in the "B" series (but not the "A") in Gillichthys, the transposed relation between "dark gray" and "medium gray" in Gambusia, the position of "white" in Girella and the position of "black" in Fundulus. It should be added, however, that in no case did one of these values deviate more than one place from its expected position in the series.

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[6] However, fishes which have become depigmented as a result of subjection to pale backgrounds will regain this pigment when transferred to black (Sumner and Wells, 1933).

In none of these fishes were the melanin values, when plotted against albedo, arranged in linear fashion. In every case except Fundulus, they were so arranged as to form a "hollow" curve. (Since Fundulus displayed such limited pigmental reactions, it may be omitted from further discussion of this subject.)

In our most extensive experiments—those upon Gillichthys, Lebistes and Girella—the whole or greater part of this curve was of the logarithmic type. In series "A" of the Gillichthys experiments this arrangement was nearly perfect. In the Lebistes experiments it held fairly well except for "black," the value for which was somewhat too low. In the Girella experiments—the most extensive of all—it held very closely except for "white," the value for which was too high, so much so as to throw it out of proper alignment in the series (Figures 1 and 2).

We have more than once pointed out the possible analogy between the relations shown in these pigmental responses of fishes to albedo and the phenomena of human sense physiology which have been generalized as the "Weber-Fechner Law." Throughout a considerable range of stimuli, it has been found that equal increments of sensation (just perceptible differences) result from *proportional* rather than equal increments of the stimulus. In Fechner's well-known formulation, Sensation = C log Stimulus.

It is commonly recognized that this is a generalization of limited application, and that at best it holds for a limited range of stimuli. Particularly is it known that the relation in question breaks down at low intensities of the stimulus, and sometimes also at high intensities (Woodworth, 1938, pp. 430 *et seq.*). Whether or not the low melanin value for "black" in the Lebistes experiments and the high value for "white" (i.e., lessened depigmentation) in the Girella experiments could be regarded as examples of the "breakdown" of any such general rule at low and high intensities of stimulation is, of course, quite questionable. For the present, we can say only that the analogy between these phenomena of pigmentary response and the phenomena comprised under the "Weber-Fechner Law," however suggestive this may be, is perhaps an entirely superficial one.

That the intensity of incident light, within wide limits, is much less effective than albedo in causing differences in pigmentation, has already been insisted upon. It is possible, however, that different species differ from one another in the degree to which light intensity is effective in this matter. In the Gillichthys experiments, it was found that 7 per cent more pigment was formed in the cabinet which was lighted by two 200-watt lamps than in the cabinet which was lighted by two 10-watt lamps. In the Lebistes experiments, likewise, a small but inconstant difference was shown, fishes from the more highly lighted cabinet (64(±) foot-candles) averaging slightly higher in melanin content than those from the less highly lighted one (0.24 f.c.). On the other hand, the rather unsatisfactory experiments with Gambusia and the recent far more thorough experiments with Girella furnish no evidence that in these fishes the wide differences of illumination resulted in any pigmental differences.[7] In the former the illuminations ranged, approximately, from 90 to 0.25 foot-candles; in the latter they were 33 and 2 f.c. respectively. It cannot be stated positively, however, that all these differences in results are due to differences of species. Several other factors (time and temperature, as well as light) varied from one experiment to another.

In respect to the amount of pigmental change brought about by these differences of albedo, we find once more that the various species employed differed rather widely from one another. Here again, we must bear in mind the reservation expressed in the preceding paragraph, although specific differences have certainly played an important role.

In counts of the melanophores in Lebistes (Sumner, 1940*a*), the mean number of melanophores in a definite area of skin was $2\frac{1}{2}$ times as great in the black-adapted fishes as in the white-adapted ones. In some lots of the same species reared by Sumner and Wells (1933), however, the differences, while not determined quantitatively, appear from photographs to have been far greater than this. In the present Girella series, we may repeat, the differences in melanin content were 2 and $2\frac{1}{2} : 1$. In the Gillichthys experiments of Sumner and Doudoroff (1937), on the other hand, the melanin content of the "black" fishes, after 87 days, exceeded that of the "white" ones by only about 30 per cent. Finally, in the recent Fundulus experiments, there were no certain differences among any of the first four albedos (black to pale gray), while the average of these exceeded the "white" value by about 42 per cent (of the latter).[8]

Before concluding, we may dispose of one possible serious criticism of our experimental procedure. It is needless to say that, when a number of fishes are kept together in bowls of the size here used, a not inconsiderable part of the visual field of every fish is occupied by its neighbors. It may be asked, accordingly, whether this circumstance may not be sufficient to invalidate any conclusions based upon the albedos of the bowls themselves.

Against any such possible objection there are rather strong arguments. It was long ago shown for flatfishes by the present writer (Sumner, 1911) that the chromatic changes of fishes are far more influenced by stimuli received from the bottom of a container than from its lateral surfaces. That this type of response is not restricted to bottom-dwelling fishes was amply shown by N. A. Wells and myself for *Fundulus parvipinnis* in 1930. In these experiments (results unpublished), two sets (four each) of 5 × 7-inch battery-jars were painted, one with the bottoms black and the walls white, the other in the reverse condition. Fishes (four in each jar) were kept for 22 days under these conditions, and were compared several times during this period in all-white jars, the two contrasted lots being poured into the latter simultaneously. With probably not a single exception, all of the fishes from the white-bottomed jars were paler than any of those from the black-bottomed ones. This despite the fact that the area of the walls of the jars, below the water line, exceeded that of the bottoms in a ratio of more than 3 : 1.

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[7] That is, if the bracketed figure in Table II is left out of consideration. If included, it would change the mean figure in the "wrong" direction, i.e., give a *lower* value for the more highly lighted fishes.

[8] Dawes (1941) reports a difference of 60 per cent in skin melanin between black-adapted and white-adapted frogs, after about five weeks. He believes that a greater change from their original condition occurred in his black-adapted than in his white-adapted specimens, since the former depart more from "the mean animal which was not kept on any particular background" (!). Dawes's claim for complete priority in the demonstration of such changes can hardly be taken seriously in view of the facts reported in the preceding pages.

In our various experiments with painted aquaria, one fish's neighbors commonly formed part of its lateral field of vision; much less commonly did they obscure its view of the bottom or any considerable part of this. Moreover, as adaptation to the background proceeded, the biological portion of this background came to contrast less and less with the remainder.

SUMMARY

The results are presented of recent experiments upon *Girella nigricans* and *Fundulus parvipinnis*, together with a comparative discussion of previous similar experiments by the author and collaborators.

In this latest series, Girella was exposed for two and for four months to backgrounds of five albedos (black, three grays and white), lighted by 100-watt electric lamps; while another set was exposed to black backgrounds only, lighted by a 10-watt lamp. In the Fundulus series, only the brighter lights were employed.

The effects of this treatment upon melanin production (or loss) were much more pronounced in Girella than in Fundulus. In Girella, the amount of melanin in the skin, after four months, was 2½ times as great in fishes from the black containers as in those from the pale gray containers. This difference in melanin content was trivial, however, in comparison with the difference in appearance of the living fishes. The latter was due, for the most part, to the transitory disposition of the pigment within the chromatophores.

The minimum melanin content in Girella was not obtained from the occupants of the white containers, but from those of the pale gray containers. The four values from "black" to "pale gray" formed, however, a descending series having a distinctly logarithmic arrangement.

In Fundulus no such arrangement was found, there being only one significant difference, that between fishes of "white" history and all of the others. The mean value of the latter is less than 1½ times that of the former.

The data from this and similar previous experiments show that in four of the five species studied, the melanin values, when plotted against albedo, form "hollow" curves, and that this arrangement, in some of the cases, is definitely logarithmic. The possible analogy between this tendency and the "Weber-Fechner Law" is discussed.

Fishes kept in black and dark gray bowls showed little change after two months. Those from the other bowls, most of all from the white, showed considerable further decreases between the 2-month and the 4-month periods. It is probable that the differences between the dark-adapted and pale-adapted fishes resulted less from increase of pigment in the former than from decrease in the latter.

No probable difference existed between black-adapted fishes kept under 100-watt lights and the same when kept under 10-watt lights, the lighting here being in a ratio of about 16 : 1.

Reasons are given for believing that the chromatic response of a fish to its background is not much interfered with by the presence of other fishes in the same container.

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THE EFFECT OF CARBON DIOXIDE AND LACTIC ACID ON THE OXYGEN-COMBINING POWER OF WHOLE AND HEMOLYZED BLOOD OF THE MARINE FISH TAUTOGA ONITIS (LINN.)

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and

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Hemoglobin combines with oxygen less readily in the presence of carbon dioxide. This influence of carbon dioxide on mammalian hemoglobin is frequently called the Bohr effect after its first observer (Bohr, Hasselbalch and Krogh, 1904). In the blood of some fish the effect of carbon dioxide upon hemoglobin is more pronounced than in mammals (Krogh and Leitch, 1919), and it is particularly conspicuous in the blood of several marine fishes (Root, 1931), in which a change in carbon dioxide tension of only a few millimeters greatly reduces the affinity of the hemoglobin for oxygen. Because the consequences have an important significance for respiratory transport, Green and Root (1933) made a theoretical study of the carbon dioxide effect in fish blood. They concluded that the effect of carbon dioxide could be described in terms of the acidification which is produced if it were assumed that acidity suppressed the acid dissociation of the fish hemoglobin and only the ionized hemoglobin combined with oxygen. According to this view, the effect of carbon dioxide could be reproduced by any other acid.

Several subsequent observations add new information pertinent to the carbon dioxide effect. The blood of a number of fresh water and marine fish, but not equally of all, is sensitive to the effect of carbon dioxide upon oxygen combination (Willmer, 1934; Black, 1940; Root, Irving and Black, 1939; Benditt, Morrison and Irving, 1941). Hemolysis of the blood of several fish was found to render their hemoglobin quite insensitive to carbon dioxide (Black and Irving, 1938), so that it appeared that the condition of the hemoglobin in the erythrocyte was essential for the special sensitivity of fish hemoglobin. Elimination of the carbon dioxide effect by hemolysis is not always complete, however, for Benditt, Morrison and Irving (1941) found that after hemolysis of the blood of Atlantic salmon the carbon dioxide effect was reduced, but still evident. The oxygen-combining power of the hemoglobin of hemolyzed blood of some marine fishes is not strikingly affected by acidification with phosphate buffers (Hall and McCutcheon, 1938). Other influences than acidity, such as the formation of carbamino compounds (Roughton, 1935) and the influence of certain ions beside those of hydrogen (Barron, Munch and Sidwell, 1937; Sidwell, Munch, Barron and Hogness, 1938) on mammalian hemoglobin can strongly affect oxygen combination.

These recent studies have emphasized the influence of factors other than hydrogen ion concentration upon the combination of oxygen with hemoglobin. It seemed desirable, therefore, to examine further the part of hydrogen ion concentration in the effect of carbon dioxide upon fish blood. The studies have been carried out on the blood of the marine fish *Tautoga onitis* (Linn.). Earlier studies had shown that the whole blood of this fish was much affected by carbon dioxide, but that there was little effect of carbon dioxide up to 75 mm. Hg pressure when the blood had been hemolyzed (Root, Irving and Black, 1939). We have subjected the hemolyzed blood of the tautog to pressures of carbon dioxide up to 500 millimeters and determined its ability to combine with oxygen. These effects are compared with those obtained when lactic acid is substituted for carbon dioxide, in both whole and hemolyzed blood, in order to determine how the effect of carbon dioxide on the hemoglobin is related to acidity. The results show that hemolyzed blood, although little affected by low tensions of carbon dioxide, reacts in a manner similar to whole blood when the carbon dioxide pressure is raised sufficiently. Furthermore, similar effects can also be produced with lactic acid.

METHODS

The methods used in obtaining blood, equilibrating it, and analyzing the gas phases were the same as those described in a previous paper (Root and Irving, 1940). Hemolysis was secured by the addition of a few drops of highly concentrated saponin solution. When lactic acid was used, a measured quantity of blood was placed in a tonometer and rapidly whirled as the acid was added drop by drop. Correction was made for dilution of the blood when determining the oxygen content of the samples.

As a criterion of the ability of any sample of blood to combine with oxygen, the blood was equilibrated with 155 millimeters oxygen (approximately air tension) at 15° Centigrade, and the percentage HbO_2 determined. The value obtained by such a procedure will be used to indicate the "oxygen-combining power" of the blood sample.

The pH of the blood samples equilibrated with carbon dioxide was determined either by use of the Henderson-Hasselbalch equation, assuming a pK value of 6.27 at 15° Centigrade, or by measurement with the glass electrode. The pH of the lactic acid-treated blood was in all cases necessarily measured with the glass electrode. A remark is necessary concerning the validity of the calculated pH values. We have plotted the logarithms of the carbon dioxide tensions against both the calculated and the measured pH values for a number of the carbon dioxide-treated blood samples. In either case, a straight line relationship obtained for almost the entire range of pH values. Although the curves for the calculated and the measured pH values were not identically placed, the calculated values being usually 0.1–0.2 pH unit higher than those measured, they paralleled each other nicely, indicating that disparity between the two could be removed by the use of a slightly different constant in the calculations.

The stability of the more acid blood samples was checked by redetermining their oxygen capacity following treatment with carbon dioxide or lactic acid. For lactic acid samples, it was necessary to neutralize the acid by an equivalent amount of $NaHCO_3$. Our experience has shown that some methemoglobin was likely to be formed in blood more acid than approximately pH 6.5, and that it was necessary to make allowance for this in calculating the percentage HbO_2 present in these samples. Methemoglobin formation proved to be more troublesome with hemolyzed blood than with the whole blood.

RESULTS

It can be seen from Figure 1 that whole blood begins to lose oxygen-combining power rapidly as the pH falls below 7.7 (carbon dioxide tension about 2 mm. Hg).

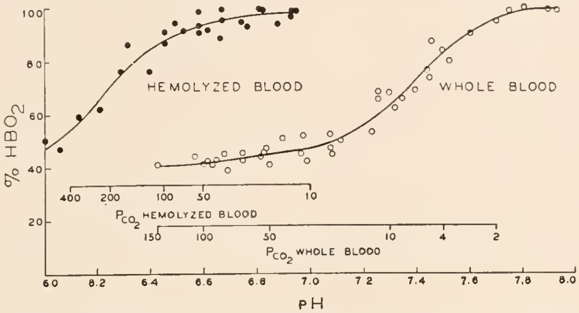

FIGURE 1. The relation between oxygen-combining power of hemoglobin at 155 mm. O_2-pressure and pH in whole and hemolyzed blood of the tautog at 15° Centigrade. The pH was modified by the addition of carbon dioxide, the approximate tensions being indicated in the graph, and was calculated by means of the Henderson-Hasselbalch equation.

Hemolyzed blood, on the contrary, shows little loss in oxygen-combining power until the pH goes below 6.5 (carbon dioxide tension approximately 100 mm. Hg). This is in agreement with Hall and McCutcheon's (1938) observation that

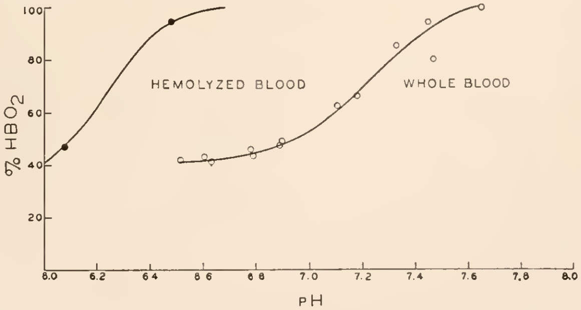

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FIGURE 2. The relation between the oxygen-combining power of hemoglobin at 155 mm. O_2-pressure and pH in whole and hemolyzed blood of the tautog at 15° Centigrade. The pH was modified by carbon dioxide and was measured by means of the glass electrode.

hemolyzed tautog blood in phosphate buffers showed little loss in oxygen-affinity through a pH range of 6.8–7.4. Below pH 6.5 it begins to lose oxygen-combining power rather rapidly. By the time 50 per cent of the oxygen-combining power has been lost the pH of whole blood is about 7.1, whereas the pH of hemolyzed blood is as low as 6. The curves remain similar in shape, and the similarity suggests correspondence in the behavior of the hemoglobin inside and outside of the cell.

The data of Figure 2, obtained by measurement of pH with the glass electrode, agree with the results shown in Figure 1. These curves cannot be exactly superimposed on the corresponding curves of Figure 1, since, as pointed out previously, the measured and calculated pH values disagreed by a constant.

The data of Figure 3 show nearly the same effect on hemoglobin when the pH is modified by lactic acid instead of carbon dioxide. This holds for both whole and hemolyzed blood. There is no great difference in the effect of the acidity produced by the two acids on the oxygen-combining power of hemoglobin in whole blood. Hemolyzed blood is also affected according to pH, although at a lower pH than in whole blood.

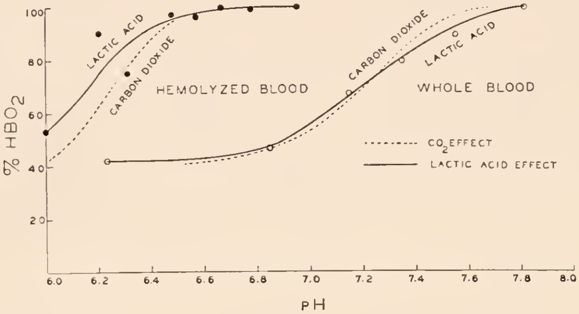

FIGURE 3. Comparison of the relationship between oxygen-combining power of hemoglobin at 155 mm. O_2-pressure and pH in whole and hemolyzed blood of the tautog at 15° Centigrade when the pH (measured) is modified either by carbon dioxide or by lactic acid.

DISCUSSION

The difference in the tension at which carbon dioxide begins seriously to affect the oxygen-combining power of hemolyzed tautog blood, as compared with the whole blood, led to the earlier conclusion (Root, Irving and Black, 1939) that hemolysis renders this blood insensitive to carbon dioxide. At that time, hemolyzed blood was treated with less than 100 millimeters carbon dioxide (maximum about 75 millimeters), and, as the present work indicates, there is no considerable loss in oxygen-combining power under these conditions. It can still be stated that hemolyzed blood is insensitive to moderately low carbon dioxide tensions; but with tensions greater than 100 mm. Hg hemolyzed blood shows the same general phenomenon as is exhibited by whole blood at a much lower partial pressure of carbon dioxide, namely, a rapid falling off in oxygen-combining power. This finding indicates that hemolysis does not so modify the properties of hemoglobin that it is incapable of responding to acidity. It suggests that, in whole blood, the pH of the cells may be considerably less than that measured in the plasma. If that view is correct, when hemoglobin is released into the plasma, the carbon dioxide tension causing a given loss in oxygen-combining power will exceed the tension required for an equivalent loss from whole blood.

To examine this view, it may be assumed that the introduction of carbon dioxide varies only pH, and that the difference in the response of whole and hemolyzed blood is only a matter of difference between plasma and red cell pH. The pH which produces 50 per cent loss of oxygen-combining power in hemolyzed blood would be that prevailing in the red cell when the whole blood also suffers 50 per cent loss. Figure 1 shows that for hemolyzed blood the pH for 50 per cent loss is approximately 6. For a corresponding loss in oxygen-combining power, the whole blood has a pH slightly above 7, and the pH of the red cell should be 6 when the pH of whole blood or plasma is 7. We have calculated the pH of the red cell from data on its carbon dioxide content given by Root and Irving (1940). The calculation was based upon determinations made at a carbon dioxide pressure sufficient to cause a 50 per cent loss in oxygen-combining power of whole blood. The calculated pH in the cell was 6.9, or 0.9 higher than in hemolyzed blood with the same oxygen-combining power.[1]

The results obtained with lactic acid, since they parallel those obtained with carbon dioxide, make it appear that the response of whole blood to carbon dioxide is essentially an acid response. The results give no indication that the anions of lactate and bicarbonate differ in their effect on the hemoglobin of fish blood.

One of the most urgent requirements yet remaining to further knowledge on the effect of carbon dioxide on fish blood is the determination of the pH inside the red cell for any given carbon dioxide pressure and pH of the plasma. The fact that hemolysis causes considerable drop in the carbon dioxide-combining power of the blood indicated that the cell was more acid than the plasma (Root and Irving, 1940), but calculations have failed to reveal that it is more acid than 0.1–0.2 of a pH unit.

We are indebted to the following individuals for assistance during this investigation: Dr. S. W. Grinnell, for measurements of pH with the glass electrode; Virginia Safford Black and Henry Brown, for technical aid in the routine analyses of the blood. We also wish to thank Dr. P. S. Galtsoff, Director, and Mr. Robert Goffin, Superintendent, of the U. S. Bureau of Fisheries at Woods Hole, for their generous co-operation in the matter of laboratory space and facilities.

SUMMARY

A study has been made of the effect of carbon dioxide and lactic acid on the oxygen-combining power of whole and hemolyzed tautog blood. The data presented show the change in oxygen-combining power of the blood as a function of pH, when the pH is modified either by the addition of carbon dioxide, or lactic acid. Both whole and hemolyzed blood lose much of their ability to combine with oxygen as the pH is lowered. The effect of carbon dioxide and lactic acid is

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[1] Prof. A. C. Redfield, who kindly read the manuscript of this paper, has suggested that there may be a change in the acid dissociation of the hemoglobin upon hemolysis. This could account for the marked difference in the behavior of the whole and hemolyzed blood toward acidity, without the necessity of assuming a large difference in pH between the plasma and the red cell. At the same time it would be quite in line with the theory advanced by Green and Root (1933) to account for the marked effect of CO_2 on the oxygen-combining power of the whole blood. We are grateful for this suggestion.

quite similar. The similarity between the effects of carbon dioxide and lactic acid suggests that carbon dioxide and anions lactate and bicarbonate have no special effect beyond the result of acidity.

The contrast in oxygen affinity of whole and hemolyzed blood is shown by the fact that hemolyzed blood must be made one pH unit lower than the calculated pH of the cells to produce the same reduction of oxygen affinity.

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INTAKE AND LOSS OF IONS BY LIVING CELLS. I. EGGS AND LARVAE OF ARBACIA PUNCTULATA AND ASTERIAS FORBESI EXPOSED TO PHOSPHATE AND SODIUM IONS[1]

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In the summers of 1940 and 1941 the writer attempted to apply the tracer technique to measure the permeability of marine eggs and larvae to inorganic ions. The γ-emission of most suitable ions prevented transmission of these ions through the mails or by express, and in consequence it was decided to try the phosphate ion. Activated phosphorus, $_{15}P^{32}$, emits β-particles, but no detectible γ-rays; the β-particles are effectively screened by ordinary packing.

The use of the phosphate ion involves on the other hand its low solubility in sea water. The ion used was predominantly $HPO_4^{=}$, since the sodium phosphate solution prepared to a pH of 7.35 was brought to a pH of about 8.0 on solution in sea water. The solubility of $CaHPO_4$, the first salt to appear on adding Na_2HPO_4 to sea water, is about 0.2 gm. L^{-1}, equivalent to 1.41 mM. No figures have been found relating to the effects of the other ions on the solubility of $CaHPO_4$. The imposed limits of solubility of phosphates made it necessary to use concentrations materially less than 1.4 mM. Here we have used 0.195 to 0.81 mM. When eggs are immersed in such dilute solutions it appears that such protoplasmic constituents as the proteins would usually be capable of combining with ions greatly in excess of the amount of the phosphate ion likely to be found in the eggs. This will be referred to in the discussion.

METHODS

Living materials. Eggs of *Arbacia punctulata* (Lam.) were obtained by removal of ripe ovaries to fresh sea water. The shed eggs were passed through gauze, and concentrated by gentle centrifugation; (2400 × gravity for 15 seconds). These eggs had a mean diameter of 72 μ, and were surrounded by a tenuous jelly 12 to 20 μ thick. This was almost completely removed by the process of concentrating eggs.

When eggs were to be fertilized or larvae reared dry sperm was collected, suspended in sea water approximately 0.5 per cent. About 0.2 ml of this suspension was added to 100 ml of sea water containing 1 ml of eggs. Fertilization tests were run in all experiments, and usually a success of 98 per cent or more was obtained. No experiments yielding less than 94 per cent success are considered here. Formation of the fertilization membrane was counted as a success. The eggs of *Asterias forbesi* (Desor.) were obtained in much the same way as those of Arbacia. But these eggs are obtained in an unripe state, in which their diameter is about 130 μ; on standing in sea water the eggs ripen, and shrink to about 120 μ. The present experiments were done with a mixture of ripe and unripe eggs. The eggs are enveloped in a jelly whose thickness is about 12 μ. This jelly is practically all removed from the eggs during their collection and concentration. In all other ways these experiments are like those with Arbacia eggs.

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[1] This work has been supported by grants from the Research Committee of the University of California and greatly helped by facilities provided by the Marine Biological Laboratory of Woods Hole. Both of these are gratefully acknowledged. In this work the writer was assisted by Dr. L. J. Mullins, Mr. A. H. Whiteley, and Mr. Aser Rothstein.

Reagents. Woods Hole sea water and water distilled at the Marine Biological Laboratory were used. Sodium phosphate was obtained through the kindness of the Radiation Laboratory of the University of California, and was thus provided as a solution either 0.210 or 0.105 M and adjusted to the pH of human blood plasma, *viz.*, 7.35. Neutron bombardment of ${}_{15}P^{31}$ transformed about one ten millionth of the atoms to ${}_{15}P^{32}$. The phosphorus thus activated was oxidized to phosphoric acid and partially neutralized with NaOH. The phosphate solutions received at Woods Hole had originally activities of 130–470 mC L^{-1}. This isotope emits β-particles of a maximum energy of 1.72 M.E.V. The activity was measured against the γ-emission of radium in equilibrium with its products. The activity at the time of each experiment was calculated using a decay constant of 0.0479 per day; the half life of ${}_{15}P^{32}$ is 14.2 days. Phosphorus containing ${}_{15}P^{32}$ will be designated as P*. A sample of radioactive NaCl was generously furnished by Prof. K. T. Bainbridge of the Department of Physics, Harvard University, and was used in two experiments. The characteristics of this isotope (${}_{11}Na^{24}$) have been described previously (Brooks, 1939).

Solutions. Eggs or larvae were immersed in solutions of this phosphate in sea water. Concentrations used lay between 0.195 and 0.81 mM, the concentrations being dictated by the radioactivity of the dilution. They are well below the solubility of $CaHPO_4$ which is 0.2 gm. L^{-1} or 1.4 mM L^{-1}. No precipitate was observed in the experimental solutions, at least during the duration of the experiments. Similarly no significant decrease in activity of these solutions was noted in the same times, the decay being negligible. The activities of these solutions at the beginnings of the experiments lay between 0.29 and 0.045 mC L^{-1}. These values lie well below the levels indicated for toxicity of Na* by Mullins (1939). This ion had been calibrated by comparison of the γ-radiations of this and radium in equilibrium with its products, and since its β-activity is about 20 times its γ-activity we may say that the toxic limit for Na* is of the order of 20 mC L^{-1}. The maximum energies for Na* (1.40 M.E.V.) and P* (1.72 M.E.V.) are comparable. It is safe to assume that so far as we know the radiation of P* in our solutions was not a factor, unless the present experiments should furnish valid evidence of such an effect.

Procedure. Three methods of exposing the material to the phosphate-sea water solutions were used: a) "common dish method." All the eggs or larvae were put into a 600 ml beaker in roughly 100 ml of solution, kept suspended by occasional swirling, and samples of 5 ml each were withdrawn at intervals. In all except the last (Exp. 13) these samples were centrifuged 20 seconds in Hopkins tubes, the solutions replaced by isotonic erythritol, centrifuged 30 seconds and the erythritol decanted. From the sediment 0.02 ml was transferred to a depression slide for measurement. The finding that much of the phosphate was removed in the erythritol solution led to a method used for one experiment (Exp. 13), in which the eggs were centrifuged once as above; centrifuged again and the last of the supernatant fluid above the eggs removed, and the eggs themselves removed until 0.02 ml was left. These eggs were cytolyzed in distilled water and transferred to a depression slide. b) "Continuous method." Not satisfied with the above procedure, we attempted a procedure in which equal portions of an egg or larvae suspension were put into coarse Buchner funnels (Pyrex 3G3) and the phosphate-sea water was slowly passed through this material, removed quickly by suction and followed by isotonic sucrose sucked through in a few seconds. The whole sample *in situ* was compared with a filter alone treated identically. This was not quite satisfying and a new procedure was devised. c) "Separate dish method." Identical samples (5 ml) of a suspension of eggs were mixed in a syracuse watch glass with 5 ml of a phosphate-sea water solution, thus avoiding the disturbances set up in the common dish method. The separate samples were collected at intervals, and to do this they were centrifuged and cleared of excess solution and otherwise treated as in Exp. 13 cited above.

In all cases the first decantate was saved, samples of 0.02 ml of this taken, and pH observed in the remainder, and attention was paid to a cloudiness which in the earlier experiments appeared to consist of fragmented eggs and possibly some egg jelly. No significance was found for the appearance or non-appearance of this cloudiness, nor of the pH which varied from 7.6 to 8.0. The activities of samples of 0.02 ml each were measured.

The sediments which in the first and third methods consisted of 0.03 ml and 0.07 to 0.10 ml, respectively, were collected with uniform centrifugation and found to contain 65 per cent eggs and the remainder of a fluid identical with the decantate. The activity of this fluid must be deducted from the observed activity, leaving an activity due to phosphate in the eggs or larvae themselves. The volumes of eggs in the samples were obtained by adjustment, or only noted and appropriate corrections were made in the calculations.

Measurements. Measurements of the phosphate content were made by a Geiger-Müller counter with a scale of eight. A definite number of impacts, usually 200, was counted, and the elapsed time noted. Comparison between the samples of eggs, supernatant, and the original phosphate-sea water solution, whose concentration was known, made it possible to translate the values from activities to concentrations. Otherwise this procedure is identical with that previously described (Brooks, 1939).

Errors in measurement. Variations in background radiation or in variations within the counter operate to change the reading. An idea of the possible magnitude of the error due to these factors can be got from seven counts of a single sample of phosphate-sea water over a period of one hour. The range of variation was 10.9 per cent of the mean. In one experiment a series of eight readings on separate samples of different fluids were repeated four hours later. The deviations here from the mean of each pair were between 0.5 and 5.5 per cent, or 0.0004 and 0.0085 mM. This confirms the above.

In addition to the counter error discussed above, there is an error in the taking of small samples. The collection of the 0.02 ml samples, used for solutions, was done in a hemocytometer pipette. Operating on seven samples of a single solution of phosphate-sea water an error of 19 per cent was found.

The egg samples were adjusted to graduations in Hopkins tubes, whose bottom cylindrical portion was graduated to 0.01 ml, an error not less than the first cited, but probably of the same order of magnitude. It is felt that observed readings may well vary up to 20 per cent, and even that *single* readings, not supported by the adjoining samples in the succession of samples, should be not seriously regarded. The levels indicated by a series of successive samples has significance.

EXPERIMENTAL DATA: ARBACIA

Eggs and young larvae. Figure 1 shows the total concentration of phosphate in successive samples of a lot of Arbacia eggs inseminated 25 minutes before the start of exposure to the phosphate. This experiment was done by the common-dish method. The early low values seem to show that no phosphate has been taken in until the time of the first cleavage, which at the temperature of 20.2° C. took place at about 50 minutes on the figure. The fact that much of the phosphate had been washed out into the erythritol solution used in washing more or less invalidates this conclusion. Cleavage can be thought of as causing an increase in permeability to the ion, or may be thought of only as interference with the washing out of the ion. In the first case we may calculate the intake after cleavage to be 0.71×10^{-10} moles cm^{-2} hr^{-1}. All values of the intake constant K are assembled in Table I. It was noted that there was no injurious effect exerted by the phosphate insofar as is shown by the comparative development of treated and untreated control eggs.

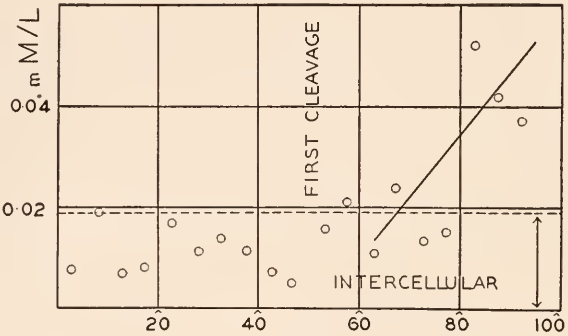

FIGURE 1. Concentrations of radioactive phosphate in samples of Arbacia eggs (ordinates) during the first 100 minutes of immersion in sea water plus 0.81 mM phosphate (abscissas). The eggs were freshly inseminated at the beginning and showed cleavage at about 50 minutes. The sloping line is the basis for the calculation of the intake constant, K. Common-dish method; washed with an isotonic erythritol solution. Temperature 20.2° C.

Figure 2 represents a similar experiment except that no washing was done, and that the eggs were inseminated only one hour before the end of the experiment. At the temperature of 19.1° the first cleavage should not have occurred during the experiment. No observation was made on this point. At the time of insemination, after one hour exposure to 0.81 mM phosphate, tests showed 94 per cent success. No great change in the general slope of the curve has been noted at the time of fertilization or elsewhere. Calculated as above we find the permeability of the egg prior to cleavage to have various possible values, depending on the degree of confidence in individual points, or the stage of the eggs. Various possibilities are shown in Figure 2 as K_1, K_2, etc., the intake constants. The value of K_1 (calculated as above) is 70×10^{-10} moles cm^{-2} hr^{-1}, a value much greater than that found in the previous figure and experiment. K_2 is about the same. Both intake rates must be attributed to the early intake as distinguished from late intake. There is usually an early maximum, sometimes two, separated by a phase of loss of this ion, from a late ion intake always slower than the first. A similar phenomenon has been noted for Nitella and the alkali metal cations (Brooks, 1939), and Spirogyra, Urechis eggs, and amebas (Brooks, 1940).

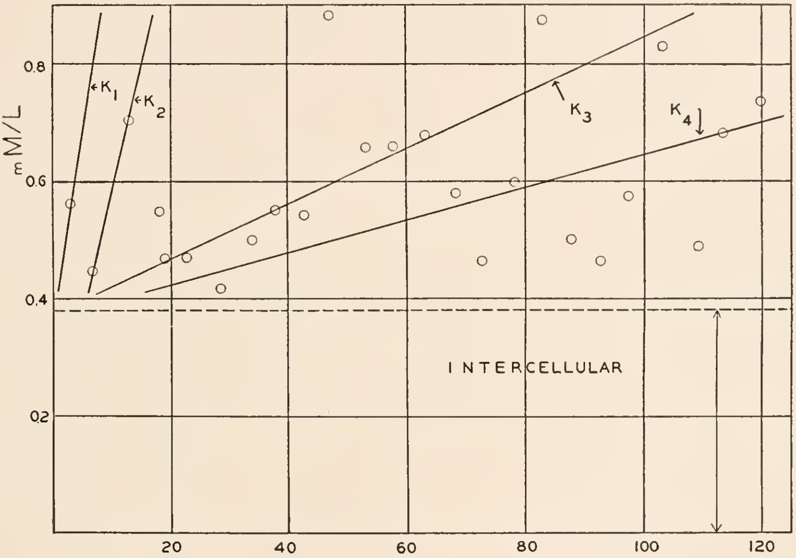

FIGURE 2. Concentrations of radioactive phosphate in samples of unfertilized Arbacia eggs (ordinates) during the first two hours of immersion in sea water plus 0.81 mM phosphate (abscissas). Four possible bases for intake constants are given. Common-dish method; no washing. Temperature 19.1°.

Apparently the washing with erythritol obscures the normal ion intake, and consequently the intake constant taken from Figure 1 is fallacious. The true late intake constants for this experiment must be about $K_3 = 3.3 \times 10^{-10}$ and $K_4 = 1.8 \times 10^{-10}$ for unfertilized and fertilized eggs.

Experiments done by other methods show the course of intake much better. Thus the results of the two experiments done by the separate-dish method are shown in Figure 3. Both experiments show the existence of an early maximum, a loss phase and a late maximum. Experiment S1 gives $K_1 = 100 \times 10^{-10}$ and $K_2 = 10 \times 10^{-10}$ moles cm^{-2} hr^{-1}, while Experiment S2 gives $K_1^2 = 33 \times 10^{-10}$ and $K_2^2 = 14.0$. The caption of Figure 3 explains the sub- and superscripts. It is apparent that these constants are not far from those found from Figure 2, in which an experiment was done with a different method (the common-dish method) but not utilizing erythritol solution washing. Washing disturbs the ion intake.

The slightly greater K values in Figure 3 may be connected with the lower concentration and radioactivity of the immersion fluid. (See Table I.)

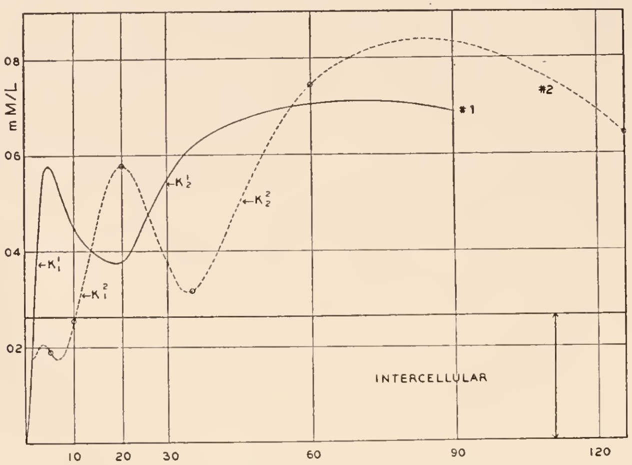

FIGURE 3. Concentrations of radioactive phosphate in samples of unfertilized Arbacia eggs (ordinates) during the first 90 or 125 minutes immersion in sea water plus 0.262 mM phosphate (abscissas) in two experiments. Tangents at four points indicated give the bases for the corresponding intake constants, namely, the early and late (subscripts 1 and 2) for Experiment S1 (superscript 1), and similarly for Exp. S2. Separate-dish method; no washing. Temperature 22°.

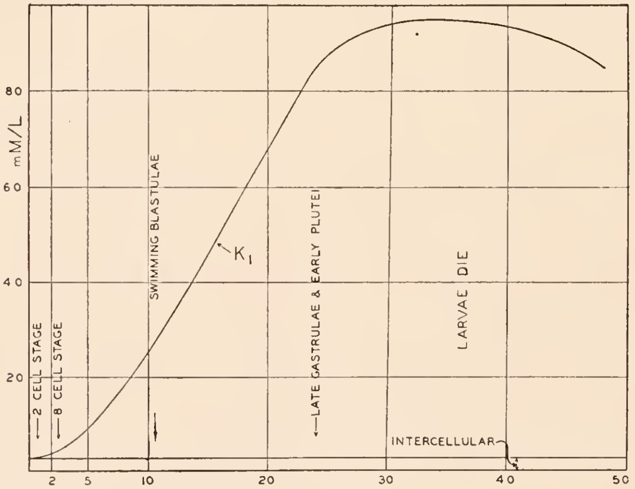

FIGURE 4. Concentrations of radioactive phosphate in samples of developing Arbacia larvae (ordinates) during the first 48 hours immersion in sea water plus 0.70 mM phosphate (abscissas). The stages attained are noted in this figure. A tangent at the indicated point gives the basis for the intake constant, K. Common-dish method; washed with an isotonic erythritol solution. Temperature 20°.

Egg to early pluteus. One experiment extended over a long period, 24 hours, in which the fertilized eggs reached the early pluteus stage. The eggs were inseminated at the beginning of the experiment, and the larvae survived in the solution more than 24 hours, but were dead within 48 hours. The stages attained are noted in the figure (Figure 4). This experiment was done by the common-dish method with washing, and apparently the early intake is obscured. But the late intake appears and can be measured.

The phosphate content of these larvae enormously exceeded that in the surrounding fluid. Soon after cleavage had started the intake accelerated, and phosphate apparently passed into the larvae at a uniform rate up until the formation of plutei. In the next 12 hours the larvae died in this experiment. The accelerated intake may be referred to the increase in surface area of the protoplasm of the larva, and modified by the deep position of certain masses of cells. Different tissues of a larva may have different permeabilities.

TABLE I

Intake and permeability constants of Arbacia and Asterias eggs and larvae for the $HPO_4^=$ *ion. The subscripts of* K *refer to the order in which these values occur during exposure. The superscripts refer to experiment number*

1 Experiment number	2 Figure number + constant	3 Stage	4 Concentration of experimental fluid	5 Radioactivity of experimental fluid	6 Intake constant	7 Permeability constant
			mM	mC/L	$K \times 10^{10}$	$P \times 10^6$
Arbacia 12	(1) K_1	2-cell	0.81	0.160	0.71	0.88
Arbacia 13	(2) K_1 K_3 K^4	1-cell	0.81	0.160	70.0 3.3 1.8	96.0 4.1 2.2
Arbacia S1	(3) K_1^1 K_2^1	unfertilized egg	0.262	0.096	100.0 10.0	36.0 3.7
Arbacia S2	K_1^2 K_3^2				33.0 14.0	13.0 5.5
Arbacia 11	(4) K_1	blastulae	0.70	0.240	3.7	5.3
Arbacia C2	(5) K_2^2	2-cell	0.195	0.045	83.0	426.0
Arbacia C1	(5) K_2^1	gastrulae	0.195	0.094	15.0	77.0
Arbacia C5	(5) K_2^5	plutei	0.350	0.055	13.3	38.0
Asterias 3	(6) K_2^3	unfert. eggs	0.175	0.246	6.5	28.0
Asterias 2	(6) K_1^2	unfert. eggs	0.175	0.260	400.0	2280.0

The intake constant can be stated for comparative purposes, by using the approximate surface area of the egg, and calculation gives us a value of 10.8 $\times 10^{-10}$ moles cm^{-2} hr^{-1}. This is not greatly unlike the values obtained in the previously cited experiments. As a matter of fact, the *superficial* area of developing larva up to the beginning of the pluteus stage should not exceed 2 or 3 times as great as that of the egg. If we modify the figure given above by using the factor of 3, we obtain a K about 3.7×10^{-10} moles cm^{-2} hr^{-1}, a figure which comports well with K_3 of Figure 2, which was 3.3×10^{-10}.

But the experiments by the continuous method give higher permeabilities: Figure 5 shows the intake of phosphate on a reduced scale of the ordinates. All three of the valid experiments show the initial peak and loss spoken of above (p. 217), but the slope of initial rise does not show intake constants higher than the later values given here. The experiment with fertilized eggs in the 2-cell stage gives a very high permeability taken from the rising phases of the curves: assuming that two blastomeres have 2 × the surface of the egg we deduce intake constant $K_1^2 = 83 \times 10^{-10}$.

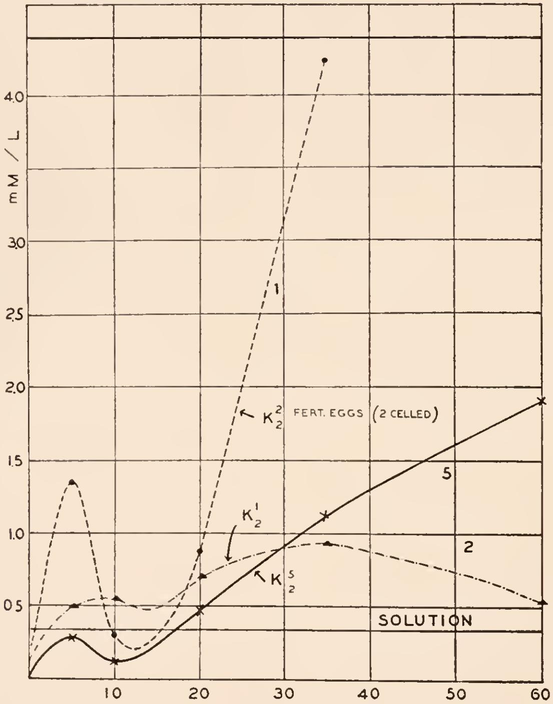

FIGURE 5. Concentrations of radioactive phosphate in samples of fertilized eggs, (1), gastrulae (2) and plutei (3) of Arbacia (ordinates) during the first 35 or 60 minutes of immersion in sea water plus 0.195 (1 and 2) or 0.350 (3) mM phosphate. Continuous method; washed with an isotonic sucrose solution. Temperatures about 22°.

Gastrulae and plutei. The curve for gastrulae (Figure 5), taken about the middle of this phase, gives an intake of about 15×10^{-10} using a factor of 3 for the probable superficial area of the gastrulae. This experiment is not very satisfactory. The plutei, allowing them a superficial area of 4× that of the egg, gives an intake of 13.3×10^{-10} given by the slope of the curve at K_2^5 (Table I).

Comparative permeability to the sodium cation and the phosphate anion. Two experiments were done to compare the permeability to sodium and phosphate ions.

Both were done before the realization that erythritol solution washing was removing the ions to a great extent. The closest approximation made on the basis of the rate of intake of these ions in the first 2 or 4 minutes, corresponding to the first peak, has been made on Experiment 5, not here otherwise shown. The rates of intake were sodium: 24.5×10^{-8}; phosphate: 0.29×10^{-8} moles cm^{-2} hr^{-1}. Compensating for the different concentrations used we obtain permeability constants for sodium: 9.8×10^{-6}; phosphate: 0.51×10^{-6} moles cm^{-2} hr^{-1} (GM $L^{-1})^{-1}$. It appears possible that the sodium cation penetrates about 17 times as easily as the phosphate dianion.

Too many considerations enter into this picture to allow drawing conclusive values. For example, the mobility of phosphate calculated from conductivity, diffusion, etc. is between those of K^+ and Na^+, e.g. the equivalent conductivity of ½ $HPO_4^=$ is 57 while K^+ and Na^+ show 65.0 and 43.4. This would be expected from the dimensions (Figure 6) of hydrated K^+ ($1H_2$), Na^+ ($7\ H_2O$) and $HPO_4^=$ (unhydrated); whereas the deduction from Figure 5 would require hydration of $HPO_4^=$ presumably about 3 H_2O to account for the lower penetrability of this ion. This envisages a plasma membrane structure which will act as an ultrafilter whose available (existent or potential) free spaces are about 7–9 Å in diameter. This corresponds generally with the results with chiefly water soluble substances, but different cells or tissues seem to vary considerably in permeability with pore size and the importance of the ultra-filter action of their plasma membranes. Possibly in passing through the plasma membrane the unhydrated ion is slowed by its divalent character, leaving only the univalent $H_2PO_4^-$, present in a relatively small proportion, to pass through the plasma membrane. Obviously much work is still needed here.

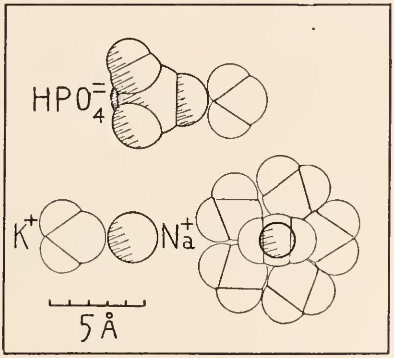

FIGURE 6. The van der Waals volumes of sodium (Na^+), potassium (K^+), and phosphate ($HPO_4^=$) ions, drawn to scale, with the mean number of water molecules attached to them in moderately concentrated aqueous solution. An ion approaching a plasma membrane would carry this hydration. Removal of this water (which may be necessary for passage) requires expenditure of work.

EXPERIMENTAL DATA: ASTERIAS EGGS

The intake of phosphate by the eggs of *Asterias forbesi* in two experiments is shown in Figure 7. In this figure as in Figures 1 to 4 a deduction was made during calculation to account for intercellular phosphate in the samples. Since these tares differ in the two experiments they are shown separately in Figure 7.

The intake curves in both experiments, like those shown in Figures 2, 3, and 5, show an initial peak ion content, followed by a loss. After this the eggs absorb phosphate at a steady, but relatively slow rate. Two intake constants have been calculated to represent the typical condition; they have been calculated from the slopes shown by $K_2^3 = 0.26 \times 10^{-10}$, and $K_1^2 = 400 \times 10^{-10}$ moles cm^{-2} hr^{-1}. K_1 is the initial, K_2 the later permeability, and the superscripts 2 and 3 refer to the two experiments. The remaining intake rates are not clearly indicated but inspection shows that they will have about the same magnitudes, respectively.

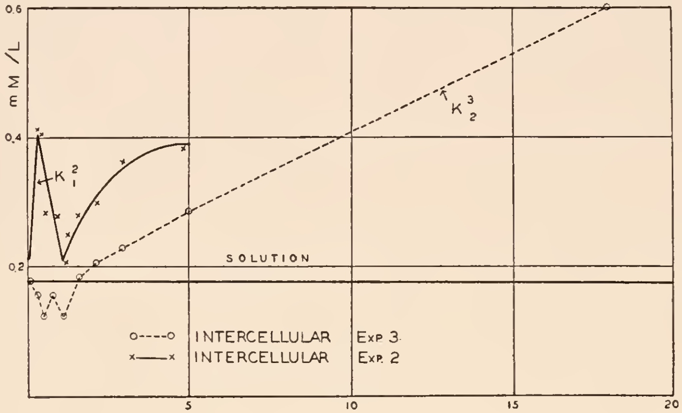

FIGURE 7. Contents of radioactive phosphate in samples of unfertilized eggs of Asterias (ordinates) during 5 (Exp. 2) or 18 (Exp. 3) hours of immersion in sea water plus 0.175 mM phosphate (abscissas). Tangents at points shown by K_1^2 and K_2^3 give the bases for intake constants for early intake in Experiment 2 and late intake in Experiment 3. Common-dish method; washed with an isotonic erythritol solution. Temperatures about 15°.

DISCUSSION

Injury. In all the experiments dealing with unfertilized eggs, tests were made of the fertility of these eggs. In all cases a success of 94 per cent or better in all stages of Arbacia; imperfect ripening of Asterias eggs led to lower degrees of success, but in no case was there any evidence of lowering by phosphate of the fertility. About 50 per cent of the Asterias eggs formed fertilization membranes after insemination.

Effect of the method used. Among other factors, some of which will be mentioned below, it appears that washing with erythritol as done in the common-dish method, was largely responsible for the apparent low permeability of the cleaving eggs of Figure 1. We forbear from comparing this with the remaining experiments cited here.

Effect of β-radiation. The assemblage of constants in Table I, columns 6 and 7, suggests the possibility that these eggs are so sensitive to β-radiation that the solutions with the higher activities yield the lowest permeabilities. Column 7 gives the permeability constants assuming that the only driving force is the concentration gradient across the plasma membrane. They are presumably more representative of the properties of the plasma membrane than are the intake constants (column 6).

Effect of phosphate concentration. Columns 4 and 7 of Table I illustrate the role of the concentration. Comparison of K_3 or K_2 of Figure 2 whose concentration is 0.81 mM L^{-1} with K_2^2 of Figure 5 whose concentration is 0.195 might be thought to indicate an effect of phosphate concentration on permeability. The continuous method deals with masses lying in streaming solutions, and the difference in permeability may be due only to diffusion factors. But here we are dealing with quite different methods, and are unsure of the cause for the difference. A similar effect is shown, however, between K_2 or K_3 of Figure 2 and K_3 or K_4 of Figure 3. But here again we find ourselves with rather different methods.

The results are so unclear that we may only suspect such a relation. Repetition of this work on an extensive scale ought to establish or negate the reality of this effect.

Permeability in different stages. Some of the evidence which can be found in Table I favors the idea that the gastrulae and plutei are less permeable than blastulae and less developed stages including the unfertilized egg (Figure 5). Blastulae appear to have about the same permeability as uncleaved eggs (Figures 2 and 4). These conclusions are far from being final.

Initial peak and losses. In Figures 5 and 6 and suggested in Figure 2 there appear an initial rapid uptake of phosphate, followed within a few minutes by a loss of this ion. This loss may proceed to levels suggesting nearly complete removal of this ion. This is so like the curve shown in previous publications for Nitella (Brooks, 1939, 1940) and Spirogyra and Urechis eggs and ameba (Brooks, 1940) to Na^+, K^+, and Rb^+ as to lead us to accept this as a reality. In these publications I have suggested that the initial rise is due to an inorganic ion exchange (Steward's "induced absorption"); no explanation has been offered for the loss of such an ion.

The rate of such an inorganic ion exchange will be affected by (a) penetrability of the radioactive ion, and its activity gradient across the plasma membrane; (b) similar properties of all available free intracellular ions available for exchange; (c) similar properties of competing ions and (d) the properties, number, and distribution of ion binding groups. If we could ascertain all of these it should be possible to account for the different rates of entrance of ions. In the case of the phosphate, both $H_2P^*O_4^-$ and $HP^*O_4^=$ being present, the presence of competing ions and the possibility of prompt combination of phosphate in metabolism make this problem still insoluble.

An explanation of the loss of ions during the so-called "loss phases" will be offered in a following paper.

Later absorption of phosphate. A frequently raised question is: how much of this ion can be combined with the surface of the egg? Toward answering this question, it is possible to calculate the number of moles of $HPO_4^=$ combined with the proteins and fats of the plasma membranes. Let us allow that one Svedberg unit of a protein, whose diameter is 30 Å, can combine with 30 equivalents of an anion. A value of 10 to 15 equivalents would probably be more applicable at the pH of the protoplasm or of sea water, and for all anions with all of which the phosphate must compete. The plasma membrane, according to Parpart and

Dziemian (1940), should also contain lipids to about ⅔ the weight of proteins, while Schmitt and Palmer (1940) show that the plasma membrane contains enough protein to form a layer 60 Å thick. These data were found for the erythrocyte. These figures are rough approximations, and the actual amounts in Arbacia eggs may differ somewhat. We use the surface 1.63×10^{-4} cm^2, and volume, 1.174×10^{-6} cm^3, as calculated from the observed diameters of these eggs. The plasma membrane is assumed to be 200 Å thick. We neglect the combining power of the lipids, which is about ½ that of proteins, so as to offset the large allowance made above for the anion combining power of proteins. A liter of eggs could combine *in their plasma membranes* with about 0.1 mM of $HPO_4^=$.

Table II shows how much phosphate got into the eggs. Only 6 out of 9 of these values exceed the calculated amounts (0.1 mM L^{-1}) which could be conceived of as combined with the plasma membrane. Nevertheless it seems improbable that this phosphate is strictly confined to the plasma membrane. So many considerations enter into the picture, all greatly reducing the theoretical figure, that it seems to be more reasonable to think of the phosphate as combining less with the plasma membrane but passing through into the cortex or interior cytoplasm. The whole egg has an ample calculated combining power to take care of much more phosphate than has entered in any of these experiments.

TABLE II

The highest recorded contents of $HPO_4^=$ *in eggs of Arbacia and Asterias during immersion in solutions of radioactive sodium phosphate in sea water* (pH 7.8–8.0)

Species	Experiment number	Figure number	$HPO_4^=$ content of external fluid	Maximum recorded $HPO_4^=$ content	Time for attaining this maximum
			mM	mM	hours
Arbacia punctulata	12	1	0.81	0.039	0.7
Arbacia punctulata	13	2	0.81	0.050	0.8
Arbacia punctulata	S1	3	0.262	0.047	1.0
Arbacia punctulata	11	4	0.7	8.3	24.0
Arbacia punctulata	C1	5	0.195	4.35	0.58
Arbacia punctulata	C2	5	0.195	0.92	0.58
Arbacia punctulata	C5	5	0.35	1.90	1.00
Asterias forbesi	2	6	0.175	0.35	0.17
Asterias forbesi	3	6	0.175	0.48	18.0

It is interesting that the experiments which show the highest phosphate tend to show most clearly the separation between the initial peak in phosphate content followed by loss of this ion. It is as though the ion had passed into the egg, and was released by a change within the egg.

But it seems probable that the phosphate is combined by metabolic processes, notably the formation of substances like hexose phosphates, base phosphates, the formation of phospholipins, and the formation of skeletal elements. This process reduces the free phosphate content of the egg, and hence favors entrance of the ion.

It will be noticed that in six of the experiments of Table II the content of phosphate notably exceeds that in the bathing solution. This condition can be attained by the operation of metabolic processes, as mentioned above, or by an organic-inorganic ion exchange, as postulated in other cases (Brooks, 1939, 1940). We feel that all three aspects of accumulation are probably operative, i.e. inorganic- and organic-inorganic ion exchange, and combination of the entering and reacting ion.

SUMMARY

(1) Eggs and larvae of *Arbacia punctulata* and *Asterias forbesi* were immersed in sea water containing low concentrations of radioactive sodium phosphate (0.175–0.81 mM) and the phosphate content in subsequently collected samples after intervals up to 48 hours was determined by measuring the β-radiation from the samples.

It was found that:

a) Phosphate was taken in often in at least two distinct periods, the first within the first half hour, followed by a loss of the ion, and secondly in the later stages.

b) The permeabilities during early absorption are generally greater than those during late absorption.

c) If it be assumed that the only driving force is the concentration gradient across the plasma membrane (a very imperfect assumption), the early permeabilities vary from 5,3 to 96×10^{-6} moles cm^{-2} hr^{-1} $(GM\ L^{-1})^{-1}$ for Arbacia and 2280×10^{-6} for Asterias. The late permeabilities of both range from 2.2 to 426×10^{-6}.

d) The maximum concentrations found in eggs or larvae of both vary from 0.050 to 8.3 mM for Arbacia, and 0.35 to 0.48 mM for Asterias.

(2) Inverse correlations are intimated between external concentration or radioactivity and permeability. The effects of radiation are discussed.

(3) The part played by the stage of the egg or larva, the effect of the methods used, the dimensions of ions, and theories of absorption are discussed.

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INTAKE AND LOSS OF IONS BY LIVING CELLS. II. EARLY CHANGES OF PHOSPHATE CONTENT OF FUNDULUS EGGS[1]

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Immersion of eggs of *Fundulus* spp. and other marine eggs in sea water containing a radioactive ion such as the phosphate or alkali metal ions reveals rapid intake, followed after a few minutes by outward migration of the marked ion and subsequent increases and decreases in content of this ion. In the summers of 1940 and 1941, during the course of work on the intake of the radioactive phosphate ion,[2] the experimental procedure was varied to obviate as far as possible the effects of handling, and special tests were made to evaluate sources of error. This paper concerns primarily the eggs of *Fundulus heteroclitus* (L.); experiments were also done on eggs of *F. majalis* (Walbaum). An earlier paper treats of similar investigations on the eggs of Arbacia and Asterias (Brooks, 1943).

METHOD

Eggs were obtained by stripping the fish. The ripe eggs of *F. heteroclitus* consist of a membranous coat, the chorion, whose mean outside diameter was 1.80 mm.; outside it is a layer of fibers which serve to moor the eggs to each other and other objects; the thickness of this membrane was of the order of 0.05 mm. Inside this lies the perivitelline fluid and the egg itself, whose mean diameter was 1.69 mm.; the egg nearly fills the chorion. Unless specially mentioned, these experiments deal only with unfertilized eggs.

Some of the unfertilized eggs were obtained in the unripe condition, and ripened gradually in sea water. The unripe eggs contain closely packed spheres of a material of refractive index higher than the rest of the egg. On ripening this material disappears either by changes in refractive index or by solution from the surfaces of these spheres or in both ways. Unripe eggs placed in distilled water become white and opaque in 5 to 10 seconds, while ripe eggs remain clear. This may be due to changes in permeability of the chorion to ions, which rapidly leave the unripe eggs, allowing precipitation of globulins or similar material inside the eggs, as suggested by Gray for trout eggs (Gray, 1932). Only in the last few of our experiments were the unripe eggs eliminated. However, there is no good evidence that the results were perceptibly affected by the presence of unripe eggs.

The eggs of *F. majalis* were slightly larger than those of *F. heteroclitus*, but

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[2] In this paper the term "phosphate ion" denotes both $H_2PO_4^-$ and $HPO_4^=$, both of which were present at the pH values used.

apparently otherwise did not differ. Experiments done with *F. majalis* are not different from those of *F. heteroclitus*. Only the latter are cited in this paper.

The freshly obtained eggs were at first used within about 15 minutes, but a few of the later experiments were started one to 1½ hours later to allow for ripening and the elimination of unripe eggs. Their viability was established by fertilization and subsequent observation. It is felt that lots of eggs giving 80 per cent or more of development are satisfactory in this work. All experiments cited gave at least this degree of fertility.

Reagents. Woods Hole sea water and water distilled at the Marine Biological Station were used. A sodium phosphate solution, 0.105 M and isotonic with human blood, was generously furnished by the Radiation Laboratory of the University of California. To make this, phosphorus element had been bombarded with neutrons, oxidized to phosphoric acid and partially neutralized with NaOH to a pH of 7.35. This isotope is described in the previous paper (Brooks, 1943).

Solutions. Eggs were immersed in solutions of sodium phosphate (containing both $H_2P^*O_4^-$ and $HP^*O_4^=$) in sea water. The concentrations used were made up to give approximately the same radioactivities. This was dictated by the sensitivity of the measuring device and the amount of egg material practicable for a single test. The limits of concentrations were 0.143 and 0.42 mM. These are well below the saturation concentration of this salt in sea water. The activities used varied between 0.17 and 0.26 mC L^{-1}. The radioactive phosphate was measured against the γ radiation of radium in equilibrium with its products.

Experimental procedures. Three general plans were used: a) The "common-dish" method in which about 50 to 100 eggs were drained and covered with 40 to 100 ml. of phosphate sea water. For each sample three eggs were withdrawn, washed in distilled water, blotted with filter paper and set aside to dry on depression slides. Such samples were collected at different intervals from one or 2 minutes up to 60 minutes and in some cases up to 24 hours. b) Hoping to obviate the mechanical disturbance involved in removing, often after tearing loose the eggs removed for successive samples, each sample was put into syracuse watch glasses. This is called here the "separate dish method." Three eggs were put into each dish in 5 ml. of sea water; to start the experimental exposure 5 ml. of sea water containing radioactive phosphate ions were added. Each of two workers handled one "series" of samples, and collected them according to an accurately timed schedule. The three eggs were transferred by a medicine dropper with minimum fluid into 100 ml. of distilled water which was then swirled. Then the eggs were removed to depression slides, and the excess fluid was removed by a capillary pipette. This whole process took 12 to 18 seconds. Two "series" of samples were thus obtained. This method yielded the most satisfying results. However, it was suspected that it still involved enough mechanical disturbance to create changes in permeability, and a method (c) was devised in which lots of three eggs each were placed in short capillaries placed in siphon tubes. This will be referred to as the "continuous method," but will not be described in detail because with this no significant changes in the results were detected.

Determinations of the phosphate content were made by a Geiger-Müller counter with a scale of eight, as described in the previous paper (Brooks, 1943).

ERRORS

Errors in counts have been shown to be usually within limits of ±20 per cent (Brooks, 1943). Differences in size of the eggs were found to lie within 14 per cent of the mean size. About 50 eggs were measured. Since three eggs were used for each sample, the error should not exceed about 5 per cent, but errors up to 14 per cent are possible. The combined error might reach 25 to 30 per cent.

Errors due to the effects of pH changes: It has been found in experiments on Fundulus embryos [3] that increases in pH to levels exceeding that of normal sea water may greatly affect the phosphate intake by the embryos. These changes in pH were produced by the addition of reagents not used in the experiments under discussion here. But it was found that the sodium phosphate, which as furnished has a pH of about 7.4, acidifies the solution to about 7.75, and by the action of decreased pH might directly or indirectly affect the result. In the case of enhanced pH, the results may be due to the precipitation of calcium or magnesium phosphates, which in turn produced solutions unbalanced toward univalent cations and hence toward the observed increased permeation of ions. This may also be due to disturbance in equilibria in solution, or to diminished H-ion. When the pH is diminished we may suspect that permeation is decreased somewhat below normal. We have no evidence that changes in pH during the progress of an experiment are responsible for significant changes in permeability, at least in Fundulus eggs. Such changes in pH seem to have been very small, of the order of 0.1 pH units, and within the error of determination as done.

DATA

Experiments on eggs. Figure 1 shows the progressive changes in one experiment in the phosphate content of whole eggs, calculated as though uniform through the whole egg. Two curves are shown each corresponding to one of two lots of eggs, and handled by one of two operators using the separate dish method. All lots were taken from a single pooled batch of eggs. The phosphate content of these samples rises during 15 minutes to a crest at about 0.20 mM L^{-1}, then drops to lower levels around 0.05 to 0.12 mM L^{-1} after 20 to 26 minutes, and thereafter tends to rise to higher levels. The curves connecting samples of single series rise generally but also show swings up and down, and the two series seem to reach these later crests and dips at different times.

Mean values calculated for these duplicate samples still show the first peak, the first dip, and to a lesser extent the subsequent increase in phosphate content, but it seems as though the individual history of a single sample is obscured by combining them to obtain the means. Until it is possible to measure the radioactive isotope concentration repeatedly in individual living eggs, not affected by mechanical handling nor desiccated (as is necessary to allow the full radiation to reach the counter cell), will it be possible to follow the uptake and loss of an ion by an individual egg. This method has been found possible in the case of Nitella (Brooks, 1939) but not so far for the larger fish eggs.

Figure 2 shows the results of two quite independent experiments, each with two series as before. They also were done by the separate dish method. They show the same phenomena as Figure 1: an initial peak followed by a dip in phosphate concentration, followed irregularly by higher concentrations. The two series of each experiment tend to follow each other at first, and then to diverge.

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[3] These experiments related to the effects of inhibitors of oxygen consumption, and have not been published so far.

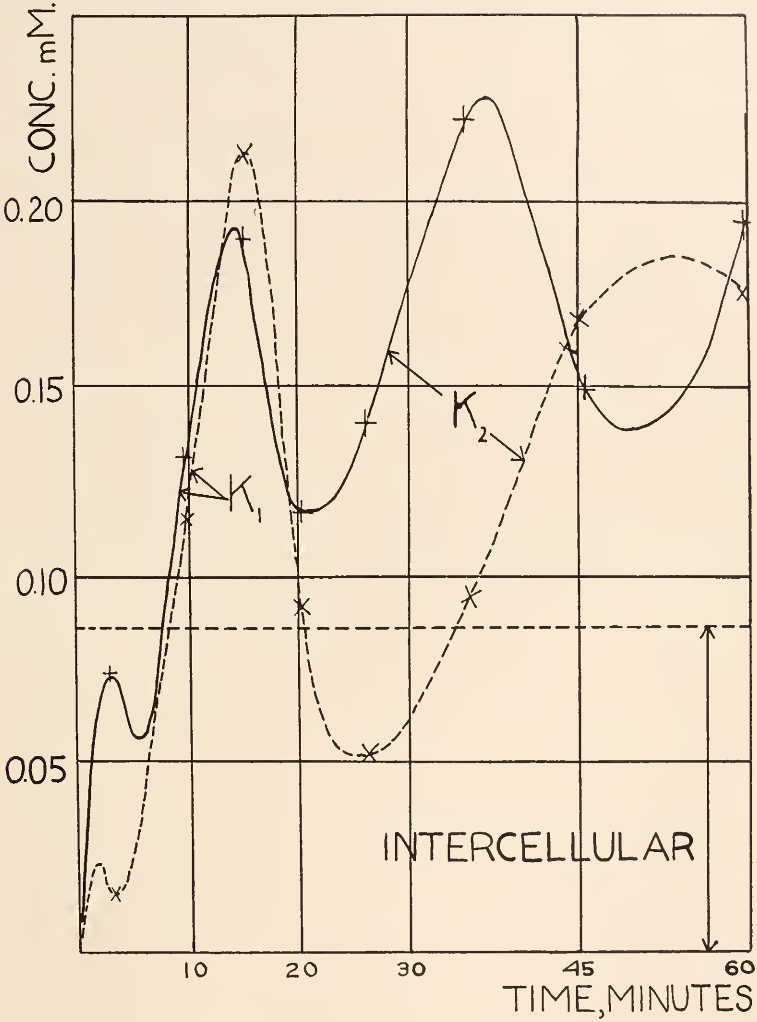

FIGURE 1. Phosphate concentrations in duplicate samples of unfertilized Fundulus eggs (ordinates) during the first 60 minutes of immersion in sea water plus 0.42 mM phosphate (abscissas). Tangents to the curves were drawn at the points indicated, and formed the bases for K_1 and K_2. Separate dish method. Temperature 17.2°.

Even the time of the first peak may vary somewhat as to the time of its appearance, viz., in one of these experiments, at 7 to 10 minutes, and in the other at 15 minutes.

Figure 3 shows an experiment done by the common dish method. Here two peaks occur at 2 and at 15 minutes, respectively. The swings are wider than in

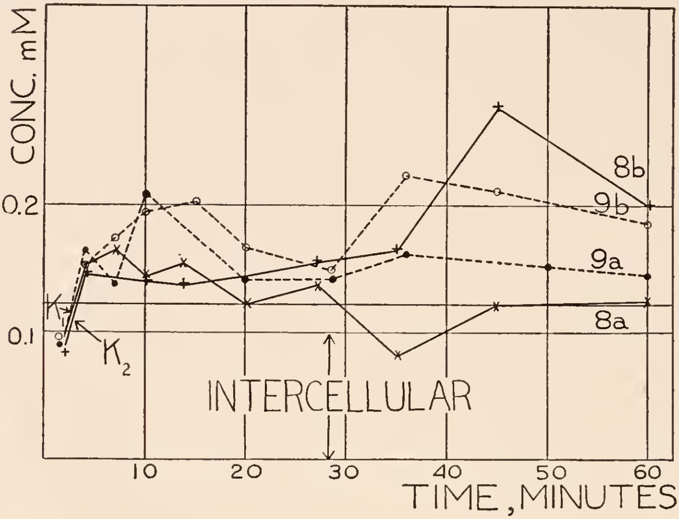

FIGURE 2. Phosphate concentrations in duplicate samples of each of two experiments on unfertilized Fundulus eggs (ordinates) during the first 60 minutes of immersion in sea water plus 0.42 mM phosphate (abscissas). The slopes of the curves at the indicated points are the bases for K_1 and K_2. Separate dish method. Temperature 17.3°.

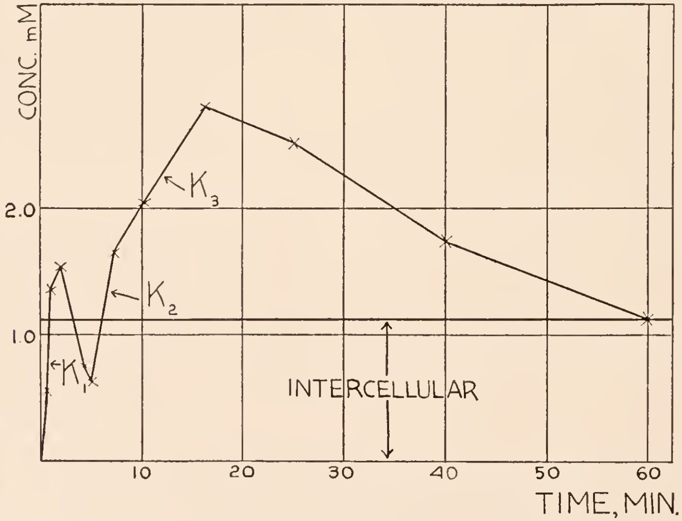

FIGURE 3. Phosphate concentrations in samples of unfertilized Fundulus eggs (ordinates) during the first 60 minutes of immersion in sea water plus 0.262 mM phosphate (abscissas). Three slopes indicated in this figure form the bases of K_1 and K_2 and K_3. Common dish method. Temperature 18.5°.

the previously cited experiments, and the peak phosphate concentration exceeds that of phosphate in the immersion fluid. The phosphate concentration in the immersion fluid was lower in this experiment, 0.262 vs. 0.42 mM for the already cited experiments. The observed results in this experiment may be due to this lower concentration.

Three experiments were done with the continuous flow method. The first such experiment showed a lower phosphate content but essentially the same as the previously cited ones (maximum 0.153 mM L^{-1}); the second shows a little less intake (maximum 0.094 mM L^{-1}); and the last gave a maximum phosphate content of 0.140 mM L^{-1}. The period for recovery from mechanical disturbance (allowed the eggs in position in flowing sea water before applying phosphate sea water) was not important: the first two continuous experiments allowed about one half-hour; the last allowed over 3 hours. The first differs from the last two, so that there is no correlation between these differences and the length of the recovery period. No cause for this rather low ion intake is apparent. It may depend upon "physiological" conditions associated with the lateness in spawning period, or possibly like those occurring in the advanced embryos.

Experiments on 8 to 10-day embryos. These experiments were done exactly like those with unfertilized eggs using the separate dish method. The immersion fluid had a concentration of 0.40 mM, and activities of 0.215 and 0.195 mC L^{-1}. The rinsed and dried eggs gave low intakes: the first had a phosphate content of 0.03 mM L^{-1} after 4 minutes of immersion and rising gradually to 0.06 mM L^{-1} after one hour (the end of the experiment); the second gave 0.035 and 0.06 mM L^{-1} after 4 and 15 minutes but gradually lost phosphate thereafter reaching 0.04 mM L^{-1} after one hour. Both experiments were done in duplicate. The two samples at a given time gave agreement within 20 per cent (= ±10%) in all but 5 of the total 20 readings of the two experiments. The observed changes were greatly in excess of the differences usually found for a single point.

The intake of phosphate in these two experiments failed to show any marked separation between early and later periods separated by loss of phosphate. The maximum contents are like those of the experiments by the continuous method cited above.

Discussion

In all of the figures it can be seen that in the later stages the phosphate content is or sometimes decreases to less than the amount needed to fill the space outside the egg itself at a concentration equal to that of the immersion fluid. Similar phosphate contents at the beginning of the experiments can be thought of as being due to incomplete diffusion of phosphate into this space, but the later occurrences seem to need special explanation.

The most obvious explanation is that marked differences in permeability of the chorion itself might lead to exclusion of phosphate in some cases, and not in other cases. But the work of Sumwalt (1929, 1933) on the potential differences across this chorion shows that potential differences are set up across this membrane when the egg is placed in salt solutions. This potential difference is not affected by the change from a chloride to a sulfate, but is halved by the change from K to Ca; equimolecular solutions of halides and sulfates give the same potential difference, but alkali and alkaline earth chlorides yield potential differences varying with the physico-chemically determined mobilities of the cation, and dilution of the bathing solution increases the observed plus potential. This has been interpreted as due to selective permeability to cations, the chorion being impermeable to anions.

It may be suggested that anions are fixed by chemosorption within the chorion, and relatively few are allowed to pass through. The chorion with a volume of about 0.50 mm^3, conceived as consisting of about 10 per cent protein of whose amino acids 10 per cent combine with anions, could account for about 2×10^{-7} equivalents of phosphate. Now, for example, the experiment given in Figure 1 shows that one egg contains newly acquired phosphate in 15 minutes to the extent of about 10^{-10} moles, or twice as many equivalents if we calculate on the phosphate diion. Figure 3 shows a larger content, but still less than 2×10^{-7}. This means that all of the phosphate found in the egg can easily be kept within the chorion itself by chemosorption or combination. This comports well with the (Sumwalt's) concept of the chorion being anion impermeable. If the protein of the chorion can combine to the estimated extent with anions the whole absorption can be thought of as combination of phosphate with the chorion. The losses following the initial and later peaks in this case might be due to changes in the combining power of the chorion, a point which will be reserved for later discussion.

It has been suggested that the radioactivity of the isotope may be responsible for this phenomenon. The β-activity of the external phosphate solution, determined by comparison with the β-activity of uranium X, was between 0.18 (exp. 14) and 0.29 mC L^{-1} (exp. 1). Muir (1942) has found that radioactive phosphate increases the viscosity of Spirogyra protoplasm (defining viscosity as resistance of chromatophores against centrifugal displacement), and alters the phosphate content when the activity of the immersion fluid was 4.0–17.0 mC L^{-1}, but has no apparent effect when its activity was 2.0 mC L^{-1}. This activity was calibrated with the same uranium X standard of about 0.25 μc, which was used in my work. Since this exceeds materially the activity of the most active solutions used in the present experiments, it seems that injury, due to β-radiation, of Fundulus eggs at this strength must be considered to be improbable until more definite experimental proof is available for the Fundulus egg.

This question may also be investigated from the basis given for the toxicity of radioactive sodium for Nitella (Brooks, 1939). Here the sodium ion, of activity equal to or less than 1 mC L^{-1}, γ-ray measured, is non-toxic. Since the ratio of β- to γ-radiation of sodium is of the order of 20 we may calculate that the β-radiation of sodium of 20 mC L^{-1} is harmless. This is only a first approximation, questions of absorption, energy of the β-rays and so on complicate the picture. The maximum energies of the β-radiations of P^{32} and Na^{24} are substantially alike, 1.72 and 1.40 MEV., so that no great difference is to be expected on this count. From this point of view also, no toxic effect of β-radiation in the present experiments it to be expected.

Exosmosis into Sea Water of Absorbed Phosphate

Procedure. About 100 eggs, obtained in the usual way, were placed in a solution of phosphate, 0.32 mM L^{-1} in sea water, and lots of 10 eggs each were taken out at intervals up to 40 or 60 minutes. They were washed in 200 ml. of distilled water for 5 to 10 seconds, transferred to depression slides, and freed from distilled water. Then 0.5 ml of sea water was added, and after three minutes the eggs were removed to another depression slide and freed of sea water as perfectly as possible with a capillary pipette, this fluid returned to the first slide. It was found that some of the phosphate came out into the washing sea water. Washing preliminary to this at this stage was avoided, so that the sum of the readings for eggs and sea water would represent the total content of the eggs when they were put into the sea water.

Readings were made on the lots of 10 eggs and on the corresponding sea water, 0.5 ml. A sample of 0.02 ml of the original phosphate-sea water had an activity of 0.24 mC L^{-1}.

Results. Figures 4 and 5 represent the results of the two experiments done. In each there are plotted the initial phosphate concentration, just before immersion in the 0.5 ml of sea water (●); a diffusion constant, K, per minute, *assuming exponential exosmosis* (○ and X); and the product of the two (+). The constant was calculated in two ways: from the decrease of phosphate in the eggs, and the increase of phosphate in the sea water. These agree within the errors of calculation.

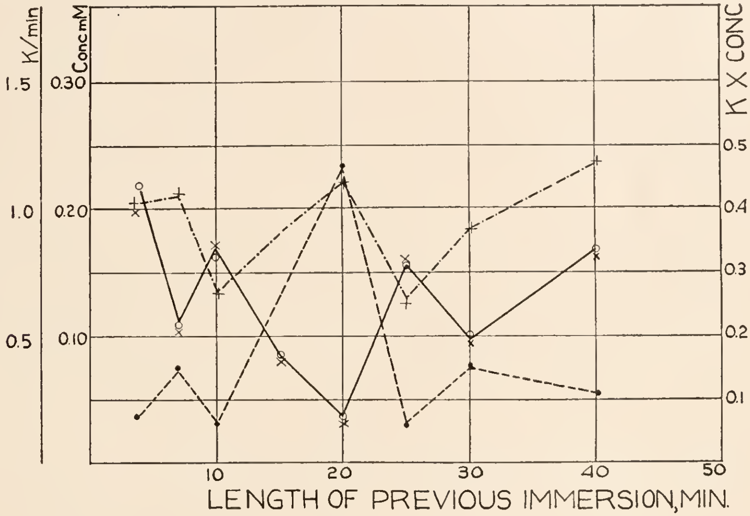

Figure 4. Rates of exosmosis from unfertilized Fundulus eggs during immersion for 3 minutes in sea water when these eggs contained phosphate in concentrations obtained as the sum of eggs plus sea water, (●) the K values calculated from measurements on eggs (○) or on the sea water (X) (ordinates) after various times up to 40 minutes of immersion in sea water plus 0.32 mM phosphate (abscissas), together with values of K X mean egg phosphate concentration (+) all plotted as ordinates.

We note that the diffusion constant is low when the phosphate concentration initially in the eggs is high, and *vice versa.* Comparing the two experiments it is apparent that the K values differ greatly and inversely to the concentrations of phosphate in the eggs. Actual figures for means of all readings were K: 0.64 and 0.21; concentrations: 0.09 and 0.26 mM; ratios 3.0 and 1/2.9. The products of concentrations by the constants do not give uniform figures, but these are more so than either constants or concentrations in virtue of their inverse relation. Their mean values for all determinations were 0.037 and 0.027, with a ratio of 1.37 in contrast to ratios of about 3 for concentrations and constants.

Discussion. These results may be interpreted as showing a) a high phosphate within a cell lowers the permeability of the cell, or b) exosmosis of phosphate is dominated principally by a constant rate of passage of this ion independent of the concentration gradient across the surface of the egg. This latter might mean that exosmosis depends on a metabolic process, or on saturation utilization of relatively few available attachment points within the chorion. The latter hypothesis is consonant with the conclusion that phosphate penetrates into and is held in the chorion only (p. 231).

The process of exosmosis can also be thought of as an ionic exchange, a concept closely related to the latter of these suggestions. For this the presence of counter ions to replace phosphate, i.e. Cl^- and $SO_4^=$, both within the chorion or the plasma membrane, and in the washing solution, here sea water, would be necessary. Data are lacking for definitive discussion; suggestions can be gotten from the work of Jenny (1936), Jenny and Overstreet (1939), Graf (1937), Hendricks (1941), and others.

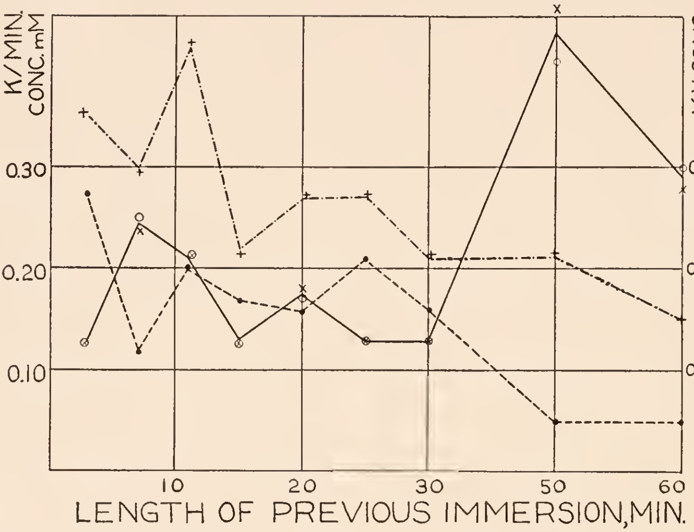

FIGURE 5. Rates of exosmosis from unfertilized Fundulus eggs during immersion for 3 minutes in sea water when these eggs contained phosphate in concentrations obtained as the sum of eggs plus sea water, (●) the K values calculated from measurements on eggs (○) or on the sea water (×) (ordinates) after various times up to 60 minutes of immersion in sea water plus 0.32 mM phosphate (abscissas), together with values of K × mean egg phosphate concentration (+) all plotted as ordinates.

Among such suggestions we may mention the possibility that replacement of univalent chloride ion by the phosphate diion may lead to dehydration of either the chorion or the plasma membrane, by simultaneous attraction of two groups or molecules (lipins or proteins possibly) by the divalent ion. Schmitt and Palmer (1940) cite the effect of Ca^{++} in contrast with K^+ in dehydrating cephalin suspensions. An effect of this type would seem to reduce the permeability of the plasma membrane. The surface layer or plasma membrane is here thought of as made up of hydrated proteins, lipins, etc. The chorion may resemble this adequately for this argument. This argues in favor of the first of the interpretations here given just above.

General Discussion

In this discussion it must be kept in mind that the phosphate may not penetrate through the chorion of the Fundulus egg during the first minutes or hours. Longer immersion surely allows this ion to reach the embryo, as had been shown by photographic tests. These demonstrate the presence of the radioactive phosphate in the skeleton of the Fundulus embryo.[4] We are not in a position to deny that the same ion passes through the chorion even during the first few minutes of immersion. For this reason we shall speak of the permeability of the egg, without attempting to trace the immediate destination of the phosphate ion.

Like the echinoderm egg (Brooks, 1940, 1943) and other materials (Brooks, 1940) which absorb cations, the Fundulus egg absorbs radioactive phosphate ions in two or more distinct periods. The intake and permeability constants are given in Table I, calculated from the slopes shown in Figures 1 to 3 at the indicated positions. The volume and surface of the ripe egg itself is used. Only minor changes in these constants would result from the use of those of unripe eggs or those of the chorion.

Table I

The intake and permeability constants for phosphate into unfertilized eggs of Fundulus heteroclitus, *together with the maximum concentration, assumed uniform, within the eggs*

Experiment number	Figure number	Constant	Concentration of experimental fluid	Radioactivity of experimental fluid	Intake constant	Permeability constant	Maximum concentration reached
			mM	mC/L	$K \times 10^5$	$P \times 10^2$	mM
S7	1	K_1	0.420	0.25	2.6	6.2	0.126
S7	1	K_2	0.420	0.25	1.16	2.8	0.134
S8	2	K_1	0.420	0.25	5.13	12.2	0.087
S9	2	K_2	0.420	0.25	4.74	11.3	0.080
1	3	K_1	0.262	0.193	18.8	71.8	0.41
1	3	K_2	0.262	0.193	6.97	27.6	—
1	3	K_3	0.262	0.193	1.11	4.4	1.69

Table I shows that the rate of intake of phosphate was of the order of 1–7 $\times 10^{-5}$, a figure which can be compared with the values for Arbacia eggs of 2–100 $\times 10^{-10}$ moles cm^{-2} hr^{-1}; and the permeability constants for Fundulus eggs were 0.03–1.69 $\times 10^{-2}$ compared with 2.2–42.6 $\times 10^{-6}$ moles cm^{-2} hr^{-1} (GM L^{-1})$^{-1}$. Asterias eggs show a permeability (Brooks, 1943) as high as 0.228 $\times 10^{-2}$. The permeability constants here, as previously, are arbitrarily based on the assumption that the only driving force is the concentration gradient into the egg. It should be noted that dimensions of this constant reduce to cm hr^{-1}, which differs from those of the diffusion constants only by cm (the diffusion constants are expressible as $cm^2\ hr^{-1}$. It has been noted (Brooks, 1943) that if the thickness of the plasma membrane is known the permeability constant can be multiplied by this thickness, becoming expressible as $cm^2\ hr^{-1}$. Here however it is doubtful that a plasma membrane is the structure involved.

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[4] Personal communication from Dr. L. J. Mullins.

The maximum concentrations found in the Fundulus eggs vary from about 1/5 to about 6 times that of the immersion fluid (Table I). In the case of the echinoderms (Brooks, 1943) we find maxima varying from about 1/26 to 22 times that of the immersion fluid. In other words, the product of these extremes is about one in each case, that is, they vary about unity. The nature of the causes for such variations is still in doubt.

The fact that the permeabilities for Fundulus eggs are so much greater than those for Arbacia and Asterias eggs suggests strongly that quite different membranes are responsible for these magnitudes. It seems probable that the values for Fundulus eggs are dominated by the chorion, while those for the echinoderm eggs are properties of their plasma membrane, or possibly of the surface layer or the whole cortex of the egg (Chambers, 1938). Just has emphasized the importance of the egg cortex (Just, 1939) but it is by no means certain that the egg's permeability is a property of the whole egg cortex.

The fact that the chorion of a Fundulus egg has so ample a possible capacity to combine, or fix by chemosorption of anions that all the content could easily be bound, makes it seem possible that relatively little phosphate passes through this membrane during the first few hours of experimentation. If this conclusion is applicable to such univalent anions as chloride, this confirms the conclusion of Sumwalt (1929, 1933).

The experiments then suggest strongly that they deal primarily with the capacity of the chorion to absorb and lose the phosphate ion and to delay its penetration into the egg by virtue of its capacity to bind this ion.

The occurrence of an early peak in ion intake, closely followed by a loss, is more or less general, and, although it has not been found by many workers, still it seems to seek a rational explanation. Pantanelli (1918) has noted such phenomena when *Valonia utriculosa*, *Saccharomyces* sp., or *Vicia Faba* and other vascular plants (whole plants with their roots in the bathing solution) are allowed to take up ions from the bathing solutions. Analysis of these fluids showed early intake within an hour or less, followed by loss and later by further intake of ions. Na^+ K^+, Mg^{++}, Ca^{++}, Ba^{++}, Zn^{++}, NO_3^-, $SO_4^=$, and $HPO_4^=$ show this in one or more cases. No radioactive ions were used, so that radioactivity cannot be blamed for this type of intake which has been widely observed. Recently Leibowitz and Kupermintz (1942) noted that *Escherichia coli* in a buffered glucose solution containing KCl would absorb K^+ strongly within the first 5 minutes, and then release it within an hour. The authors felt that this behavior was associated with the formation of a polysaccharide, but the brief communication did not allow convincing proof of this point.

In this connection it is of interest to note that amides and urea decrease the viscosity of the ectoplasm (cortex) of *Spirogyra* sp. followed by return to about the initial value. This was followed by repeated wide variations in viscosity (Northen, 1940). Failure to maintain the normal position of the chloroplasts during centrifugation was considered to be evidence of decreased viscosity. This whole picture resembles that of the ion content of living cells during ion absorption.

This parallelism suggests that the ion content is intimately connected with the state of the proteins. Northen (1940) speaks of "protein dissociation." It may be that the release of fatty materials from the Arbacia egg by the action of NH_3 (Heilbrunn, 1937), the bringing out of the double diffraction by lipins in the Golgi apparatus of snail spermatocytes by the use of chrysoidin, an amino containing dye (Monné, 1939), the similarity of the action of the K and NH_4 ions and acetyl choline in breaking down the cell membranes at nerve synapses (see in this connection Mann, Tennenbaum, and Quastel, 1939) and the general toxicity of NH_4 salts all reflect physico-chemical changes in proteins, presumably globulins or possibly nucleoproteins. Such a change of state by a protein is shown when the rodlets of tobacco mosaic virus, revealed by the electron microscope technique, lose their ordered configuration when they are suspended in NaOH or NH_4OH containing solutions (Stanley, 1935; Stanley and Anderson, 1941). The behavior of this nucleoprotein is due to the presence of NH_4^+ or NH_3, and Na^+ or possibly the OH^- ion. Inactivation, which is related to this type of conversion of tobacco mosaic virus, occurs also in urea solutions (Stanley and Lauffer, 1939), and in alkaline suspensions produced by addition of NaOH (Stanley, 1935).

At least one of the alkali metal ions appears to be effective, and if all such ions have similar properties in this case, as experiment shows in similar cases, then the exposure of living cells to unbalanced solutions rich in such cations as Na^+, K^+, Rb^+, Cs^+, NH_4^+, and probably the organic cations such as choline, guanidine, amines, urea, the basic amino acids, etc. would entrain a change in the protein molecules ("conversion"). This, by analogy with the observed effects on viruses, would probably decrease the capacity of these proteins to participate in the molecular structure of the plasma membranes. The loss of elongated molecules should deprive the plasma of essential, mechanically necessary components. This effect might be produced directly, or by releasing NH_3 or NH_4^+ which are continuously present in any synthesizing cell, and allowing them to affect the plasma membrane secondarily. Organic cations might well be an equally important factor (Hendricks, 1941). The mutual replacement of ions has been studied carefully by soil students. Gieseking and Jenny (1936) found that the NH_4^+ ion is very easily replaced by such ions as Na^+ and K^+. The alkaline earth ions are more resistant to replacement, and their function can well be pictured on this basis. For example, the presence of enhanced proportions of these ions should stabilize the plasma membrane, and delay or prevent the conversion of proteins such as we picture.

This breakdown in the plasma membrane is envisioned as marking a change from a membrane permeable to ions by ionic exchange, that is, a membrane having pores of about 7–9 Å diameter (Brooks, 1943), presumably left between protein molecules and preponderantly plus or minus charged, to a membrane which allows ion pairs to diffuse through it, i.e. with larger and little charged pores ("mass movement" of Fenn, 1940). When a cell is placed in a solution containing suitable cations or anions we find that the ions of this solution are soon seen in the protoplasm itself (e.g. Brooks, 1940), and tend to accumulate in excess of the concentrations in the bathing solution. Soon, however, another process supervenes and results in the movement of same ions (and perhaps others) out of the protoplasm into the bathing solution. The first can be accounted for by ionic exchange (by segregated cation exchange and anion exchange) utilizing as counter ions such things as NH_4^+ or organic cations, and any of the organic anions or Cl and phosphates, all of which are known to occur or are produced within living cells generally. The second process is the loss of these accumulated ions by allowing the free diffusion of salts to occur, involving the simultaneous movement of the oppositely charged ions. The observed repetition of these gain and loss phases strongly suggests that the fundamental changes in the proteins of the protoplasma membrane and of the cortex can be reversible, or that converted proteins are replaced by similar unconverted proteins from the deeper lying regions in the cell. Newly made proteins may also serve in this replacement. This hypothesis accounts qualitatively for the observed course of ion absorption where accumulation and loss of ions occur. The loss phases might well be absent in the cases where they were not observed either because of the absence of active intracellular cations, or because of a more stabile plasma membrane.

This idea is also of importance in a case like the Fundulus egg. We have noted that unripe eggs have a chorion which may be easily permeable to ions. But on ripening the chorion should attain sufficient closeness of their fabric to allow some degree of ion accumulation. Like the plasma membrane it may during an experiment suffer a change in its proteins and allow loss of salts by mass movement. Such a similarity between the chorion and its parent substances is adequate to account for the similarity of the gain and losses of ions when Fundulus eggs are placed in suitable salt solutions.

SUMMARY

Unfertilized eggs of *Fundulus heteroclitus* were immersed in sea water containing low concentrations (0.193 to 0.25 mM) of radioactive sodium phosphate and the phosphate content in subsequently collected samples after the intervals from one-half minute to one hour was determined by measuring the β-radiation from the samples.

It was found that:

(a) The phosphate ion was taken in during two or more periods, separated by periods during which the ion was lost.

(b) During the first rise the permeability was high ($6.2–71.8 \times 10^{-2}$ moles cm^{-2} hr^{-1} $(Gm\ L^{-1})^{-1}$) while the later rises have lower permeabilities ($2.0–4.4 \times 10^{-2}$).

(c) The maximum concentrations found in these eggs varied from 0.080 to 1.69 mM or about 1/5 to about 6 times that of the immersion fluid for the respective experiments.

The great rates of penetration (permeabilities) found, considered with the relatively high combining power of the chorion has led to the tentative conclusion that during the first hour or so of such experiments very little phosphate penetrates through the chorion to the egg cell itself.

A tentative theory as to the nature of the processes leading to intake and often accumulation of an ion, its subsequent loss, and repetition of this cycle is proposed. It depends on the assumption that proteins suffer reversibly or irreversibly a "conversion" during ion intake, and that this is due to the action of the ions concerned.

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BILIARY AMYLASE IN THE DOMESTIC FOWL

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Although the question of the secretion of a biliary amylase by higher vertebrate animals is discussed extensively in earlier physiological literature it has attracted little attention in recent years. The general assumption among physiologists is that bile has no enzymatic function in the processes of digestion. Loehner (1929) presented a critical discussion of the earlier literature and recorded the results of his own investigations in which he was able to prove the presence of an amylase in the bile of cattle and sheep. Fossel (1931) demonstrated that this amylase was hepatic in origin and not due to contamination with amylase from the intestine. There is little information on biliary amylase in birds. Jacobson (1856) reported its presence in the domestic fowl, domestic duck, and domestic goose. Because of the methods used by him the probability of bacterial action as the cause of amylolysis is not precluded. Stresemann (1934) stated that amylase is found in the bile of the domestic goose. When preliminary investigations by the author gave positive results he decided to conduct further experiments which would give information concerning the relative amylolytic activity of the bile and the general occurrence of biliary amylase in the domestic fowl.

Materials and Methods

White Leghorns, Rhode Island Reds, and Barred Plymouth Rocks from the stocks of the Department of Poultry Husbandry were used as experimental animals.

Bile from the gall bladder was obtained by removal of the intact organ. The bile was then drained directly into sterile vials (toluol added) by means of a small incision in the wall of the bladder.

Bile was also obtained from the hepatic duct which leads directly from the liver to the intestine. To secure this the bird was anesthetized with nembutal or ether and placed on its left side. An incision was made on the right side posterior to the last rib and parallel to the sternum at such a level that the duodenal loop could be reached. This loop was then drawn to the opening made by the incision, thus exposing the cystic, hepatic, and pancreatic ducts. A glass cannula with an inside diameter of about 0.7 mm. and a slight enlargement about 5 mm. from the end was inserted through a small incision in the duct and tied in place. The purpose of the slight enlargement near the end of the cannula was to prevent its slipping out of the duct after it had been tied in place. A gum rubber collecting chamber, 15 cm. long and 1.5 cm. in diameter, containing a few drops of toluol was then attached to the free end of the cannula. Usually the collecting chamber was attached to the cannula before it was inserted into the duct. The chamber was carefully placed among the loops of the intestine and the incision sutured. After 24 hours the bird was killed and the chamber removed. Seven successful operations yielded 4 to 10 ml. of bile each. The principal difficulty encountered and the reason for most of the failures was the clogging of the cannulae due to their necessarily small diameters.

Pancreatic juice was obtained from the largest pancreatic duct by means of a similar operation. The recovery of the birds was surprising. In more than 30 operations there were only three deaths, two during anesthesia and one from hemorrhage.

Bile and pancreatic juice were used immediately for tests or were stored at 1° C. with toluol until used. No differences could be detected between the fresh and stored samples.

Amylolytic activity was estimated quantitatively from the reducing sugar produced by the action of the bile on one per cent boiled starch substrate (toluol added) with 0.01 M phosphate buffer and 0.02 M NaCl at 40° C. Quantitative estimations of reducing sugar were made according to the method of Shaffer and Somogyi (1933). The only modification of this method necessary was the extension of the heating time of the digest sample with the copper reagent. An arbitrary "amylase unit" was established for convenience in expression of results. This unit is defined as that amount of amylolytic activity which will in one hour produce 25 per cent of the maximum amount of reducing sugar from one ml. of one per cent boiled starch at pH 7.10 (0.01 M phosphate buffer) with 0.02 M NaCl at 40° C. Trial digests were made to establish the concentration of bile which would give a linear relation with time for a period of about one hour after the addition of the bile to the substrate.

Similar digests were made for qualitative tests. Reducing sugar was detected by Benedict's qualitative test. All tests whether quantitative or qualitative were made within one hour after the addition of bile to the substrate.

Experiment 1

In order to ascertain whether or not the biliary amylase was hepatic in origin, bile was collected from the hepatic duct by means of a cannula thus excluding the possibility of contamination by amylase from the intestine. Samples of hepatic duct bile from six birds were compared quantitatively with the gall bladder bile from four of these birds as well as with gall bladder bile from 11 others. The

TABLE I

Relative amylolytic activity of bile and pancreatic juice

Group	Juice	Number of birds	Activity in amylase units*		Optimum pH†
			/ml. juice	/mg. dr. weight	
A‡	Gall bladder bile	15§	15–90	70–500	7.1 –7.2
B‡	Hepatic duct bile	6	10–30	200–700	7.1 –7.2
C	Pancreatic juice	3	800–6000	4000–600,000	7.05–7.15

* Arbitrary unit for comparative purposes. See text.

† With 0.01 M phosphate buffer and 0.02 M NaCl.

‡ Groups A and B have 4 birds in common.

§ Samples from 10 of these pooled and treated as a single sample.

samples from ten of the latter were pooled and treated as a single sample. The data recorded in Table I show that the hepatic duct bile contained amylase in amounts comparable to that in the gall bladder bile thus proving its origin in the liver. Comparisons were made on a volumetric as well as dry weight basis. For the purposes of studying relative activity, samples of pancreatic juice from three different birds were analyzed quantitatively. The relative amylolytic activities of bile and pancreatic juice as recorded in Table I reveal that the action of bile on starch is small when compared with that of pancreatic juice.

The optimum hydrogen ion concentrations for the amylolytic actions of gall bladder bile, hepatic duct bile, and pancreatic bile were determined by buffering the substrate to various hydrogen ion concentrations with 0.01 M phosphate buffer in the presence of 0.02 M NaCl. The optimum hydrogen ion concentration as recorded in Table I agrees well with the accepted value for pancreatic amylase (pH 7.1) in the presence of the same concentration of NaCl (Tauber, 1937).

Experiment 2

The general occurrence of amylase in the bile of the domestic fowl was studied with qualitative tests on 50 birds in three age groups as shown in Table II.

TABLE II

General occurrence of biliary amylase in the domestic fowl

Source of bile	Age of birds	Number of birds	Occurrence of amylase in bile*	
			present	not present
Hepatic duct	adult	6	6	0
Gall bladder	adult	20	19	1
Gall bladder	8 weeks	12	10	2†
Gall bladder	4 weeks	12	7	5

* Digest with 0.1–0.3 ml. bile and 1.2 ml. starch substrate with conditions as described in text.
† Both contained traces of amylase.

Samples which showed only traces of amylolytic activity were regarded as negative. Indications from these tests are that its occurrence is irregular among younger birds.

DISCUSSION

Since bile obtained by the cannulation of the hepatic duct contained amylase the possibility of its presence being due to contamination by amylase from the intestine is eliminated. This is in agreement with the results obtained by Fossel (1931) in cattle and sheep. His observation that due to reabsorption of water the concentration of amylase in gall bladder bile is higher than that of hepatic duct bile appears to be supported by the results of these experiments. Loehner's observation that the amylolytic activity of the bile is not great when compared to that of the pancreatic juice is certainly supported by the data obtained in this investigation. However, the function of biliary amylase in the processes of starch digestion should not necessarily be designated as unimportant until the relative rates of secretion of bile and pancreatic juice are known. No information concerning this is available for the domestic fowl at the present time.

Since the liver is generally supposed to contain a starch-splitting enzyme, "glycogenase," the question arises as to the possibility that biliary amylase and "glycogenase" are identical. The author has no evidence concerning this. Any consideration of this possible identity must take into account the conclusion of Cori and Cori (1938) that glycolysis in the liver is due to the action of an enzyme system rather than to the action of a single enzyme, "glycogenase," as formerly supposed.

SUMMARY

The bile of the domestic fowl contains an amylase which is secreted by the liver. Its optimum hydrogen ion concentration is similar to that of pancreatic amylase. The amylolytic activity of pancreatic juice was found to be 10–800 times that of bile.

Acknowledgments

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ACCELERATION OF CLEAVAGE OF ARBACIA EGGS BY HYPOTONIC SEA WATER

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During the summer of 1938, a series of experiments was undertaken at Dr. Robert Chambers' suggestion, with a view to determining something of the mechanism by which decreased tonicity slows mitosis. In the dosage-rate curves there was noted an aberration which seemed to indicate an acceleration of cleavage in slightly diluted sea water. Detailed investigation showed the acceleration to be real,[1] and the results are presented here.

I am indebted to Dr. Robert Chambers for continued helpful advice, and to Dr. Alvalyn Woodward for sharing laboratory space supplied by the Faculty Research Fund of the University of Michigan during the summer of 1941.

MATERIALS AND METHODS

The eggs of *Arbacia punctulata* were used throughout. For each experiment, the washed, concentrated eggs were measured into control and hypotonic solutions in quick succession. All eggs were examined after 24 to 48 hours for normal development. Hypotonicity is represented in percentage of normal sea water: 96 per cent being 4 parts of distilled water in 96 of sea water. Both concentration and time of exposure were varied experimentally.

The solutions and eggs were brought to a uniform temperature before mixing, and the temperature maintained with a water bath. There was no difference among the solutions measurable within a tenth of a degree Centigrade, and the positions of the flasks were rotated to prevent favoring of any one by an imperceptible temperature difference. In some of the earliest experiments, in which a small water bath was used, the temperature rose as much as 3° C. during the course of the experiment, but frequent measurements of the solutions were made to insure an identical temperature in the different solutions. The temperatures employed ranged from 18.5° to 23° C., being kept close to that of the running water in the tanks to avoid temperature shock in transferring the eggs from the sea urchins to the solutions.

It should be emphasized that alteration in rate of cleavage always refers to a comparison of experimental and control lots of eggs from a single female, run at the same time and subject to the same variations in temperature or other environmental factors. To check on variability in the method, experiments were run with sea water in all flasks. The curves thus obtained from these six identical controls were exactly superimposable, and treated statistically showed no significant differences. Besides sea water controls, isotonic controls were also used in some experiments to check against the effect of electrolyte concentration. Isotonic sodium chloride was used as a control for total electrolyte content. These solutions were introduced in the same quantities as distilled water in the experiments with tonicities of 96 per cent and 95 per cent.

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[1] A search of the literature revealed that this accelerating effect of hypotonicity had received considerable attention, but no experiments upon the cleavage rate of marine eggs had been reported. Not until the work upon Arbacia eggs had already been reported (Cornman, 1940) and the full account was ready for press was it discovered that the acceleration of cleavage in Arbacia eggs had been extensively studied ten years earlier in the physiology class at Woods Hole under Dr. M. H. Jacobs' direction. The effect had been noted before that by Dr. Walter E. Garrey.

In the crucial experiments the pH of the media was checked with a glass electrode at the beginning and at the end of the experiments. Between the concentrations 100 per cent and 88 per cent sea water, the range in which acceleration of cleavage occurs, there was found to be a drift of only 0.1 pH unit toward alkalinity upon dilution, and no further change during the course of the experiment. The natural buffers of sea water were adequate to prevent any greater change in pH.

Comparison of the rates of cleavage was made by statistical analysis of samples fixed at successive intervals of two, three, or five minutes. This was done by pipetting samples from each of the control and experimental solutions, into one per cent formaldehyde, all within 10 to 15 seconds of each other. In each of these samples, 200 eggs were counted. In order to obtain samples that were as representative as possible, the experimental solutions were stirred after each sampling, and the killed eggs were thoroughly mixed before they were transferred to the counting slide. In counting the eggs, a regular pattern was followed which covered the entire slide. From these samples, the percentage of eggs cleaved within each period was determined. Arranged in a frequency table, these percentages supplied the distribution, mean, and median of the time between fertilization and cleavage. In these statistical calculations, the percentage of eggs cleaved during the period between the moments of sampling constituted the frequencies of the classes, and the times at which the samples were taken constituted the class limits. Thus, the data could be handled by conventional statistical methods. Although not entirely satisfactory, it gives a preliminary estimate of the validity of results. Complete analysis involves special statistical problems which cannot be dealt with here. In all, over 500,000 eggs were counted.

OBSERVATIONS

The results of typical experiments are represented graphically in Figure 1. Connecting the cleavage percentages are steep ogives, tapering somewhat more near the 100 per cent cleavage mark than near zero. Experiment I–12 (Figure 1) show a series of curves revealing a clear-cut acceleration in 96 per cent and 92 per cent sea water, no acceleration in 88 per cent, and retardation in 84 per cent.

In general, acceleration of the first cleavage was found to occur in concentrations between 100 per cent and 88 per cent sea water. Acceleration was obtained with treatments beginning anywhere from four minutes after insemination, up to the diaster of the first cleavage. There was no sharply defined peak of acceleration at any one tonicity, but acceleration in 96 per cent was most frequently the highest. Moreover, the maximum acceleration for any single experiment occurred at 96 per cent: a statistically calculated acceleration of 3.36 minutes, which, referred to the control mean cleavage time of 65.36 minutes, represents a shortening of the cleavage time by 5.1 per cent. Accelerations obtained varied from this

5.1 per cent to zero. Particularly during the summer of 1941 the eggs showed little response to hypotonicity, although the experimental procedure was the same as previously used. It should be emphasized that the variation was between zero and statistically significant accelerations. In the several hundred experiments there were few decelerations obtained in 98 per cent to 94 per cent sea water, and none of these was significant insofar as the statistical methods employed could show. In the range 92 per cent to 88 per cent, on the contrary, some experimental sets were retarded, while others were accelerated. This possibly represents a different threshold of response among the different sets. Dilutions to 84 per cent and more always gave decelerated cleavage, as did all hypertonic solutions.

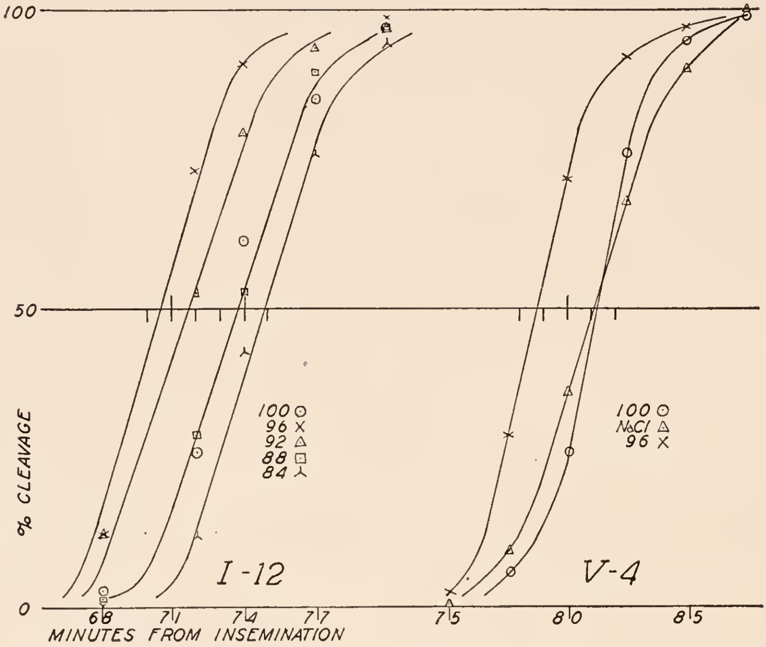

FIGURE 1. Typical curves for first cleavage, showing a moderate hypotonicity effect, the amount of scattering about the ogive, and the slight variations in slope. The curves are smoothed by eye in accordance with the general trend of a large number of curves.
I-12: Cleavage curves for 84 per cent to 100 per cent sea water. They show acceleration in 96 per cent and 92 per cent, no effect in 88 per cent, and retardation in 84 per cent. The acceleration in 96 per cent, determined statistically, was 2.85 ± 0.43 minutes. Treatment begun 14 minutes after insemination. Temperature 19° C.
V-4: Cleavage curves for 96 per cent and 100 per cent sea water, and for the isotonic sodium chloride control for 96 per cent. There is a decided acceleration in the hypotonic sea water, but none in the isotonic control. The statistically determined acceleration in 96 per cent was 2.31 ± 0.36 minutes; in the isotonic control, −0.04 ± 0.41 minutes. Treatment begun 13 minutes after insemination; temperature 18.5° C.

At 19° to 20° C., cleavage between 5 per cent and 95 per cent is completed in from four to 20 minutes in normally developing eggs. (The spread for the steep section of the ogive in experiment I–12 is about 8.5 minutes.) There are much smaller differences of spread within a single experiment (Figure 1). These differences in slope have not been analyzed for significance.

Changes in electrolyte content and electrolyte balance caused no acceleration. Cleavage curves for 96 per cent sea water, and sea water diluted to 96 per cent by isotonic NaCl, are compared with cleavage in 100 per cent sea water in Figure 1, experiment V-4. Both maltose and sucrose isotonic controls retarded cleavage slightly, but probably not significantly. Lillie and Cattell (1929) found no considerable alteration of cleavage rate, even with electrolytes reduced to 60 per cent with isotonic sucrose.

When eggs remain in the hypotonic solutions, the acceleration is not duplicated in the second and third cleavages. Fewer figures are available for these cleavages, but they indicate definitely that after acceleration of the first cleavage, subsequent cleavages are not accelerated to the same extent. Also, if the first cleavage does not respond to hypotonicity, the second cleavage does not. However, the second cleavage will sometimes respond to hypotonicity if the eggs are immersed after completion of the first cleavage. The acceleration is not as marked as that obtained in the first cleavage, reflecting, perhaps, the changed condition of the egg, and the shorter time available for the hypotonicity to act.

Discussion

The amount and consistency of acceleration obtained show clearly that cleavage of Arbacia eggs proceeds faster in hypotonic sea water than in normal sea water. However, dilution of sea water has effects other than reduction of the tonicity, so it remains to be shown that tonicity, and not some other dilution effect is responsible for the acceleration. Electrolyte concentration is one of these factors which must be distinguished from simple osmotic activity, since the electrolytes in sea water affect cells in many ways other than by their osmotic pressure. To test this possibility, it is a simple matter to reduce the electrolyte content without reducing the tonicity, by introducing isotonic nonelectrolytes into control solutions. Maltose and sucrose were used in these controls. The results with both were the same: a slight retardation of doubtful statistical significance. Electrolyte balance may also be involved to a slight degree, since some salts occur in sea water in much lower concentrations than do others. Sodium chloride is the most plentiful, and so diluting sea water might have the effect of changing the salt balance in favor of NaCl, particularly if one of the less abundant salts were brought near a physiological threshold of the Arbacia egg. If this is the case, then diluting sea water with isotonic NaCl should effect an even greater NaCl preponderance without any tonicity change. In controls diluted, no significant difference from the sea water control was in evidence. Unless we assume that sugars or sodium chloride in some way counteract a stimulation caused by changes in electrolyte content or salt balance, we must conclude that electrolyte alterations play no part in the acceleration of cleavage by dilution.

There remains the question of pH effect, since a very slight trend toward alkalinity occurred in the range of dilutions which produced acceleration. This shift amounted to 0.1 pH unit upon dilution to 90 per cent, from which interpolation gives a 0.05 unit shift for the 96 per cent and 95 per cent dilutions. While it is extremely small, this pH shift must be taken into consideration, because a number of investigators have found an acceleration of cleavage in sea water made more alkaline. There is by no means unanimous agreement upon this acceleration by alkalinity. Jacques Loeb (1913) concluded that the natural hydroxyl ion concentration is optimum for the development of sea urchin eggs, although he had earlier reported (1898) that addition of sodium hydroxide accelerated development. Of the several papers reporting acceleration of cleavage with increase in pH, the work of Smith and Clowes (1924) with Arbacia eggs is the most applicable, since they determined the precise pH values involved. In their Figure I, a pH increase of 0.05 corresponds to an acceleration of less than one per cent. Even part of this small increase must be a hypotonicity effect because 0.02 N NaOH was used to increase the pH, whereas an isotonic concentration would be around 0.5 N. So while tonicity cannot account for the marked acceleration obtained in basified sea water, nor pH for the acceleration obtained with slight dilutions, there remains the possibility that the results of Smith and Clowes, and those presented here, represent to a slight degree combined effects of pH and tonicity, in vastly different proportions.

Since the accelerations in the various hypotonicities do not form a smoothly graded series when different experiments are compared, it should be emphasized that these results were obtained under a variety of experimental conditions. Temperatures, chosen to conform with that of the incoming sea water to avoid temperature shock, ranged from 18.5° to 23° C. Eggs from different sea urchins vary in response, and there possibly is a seasonal difference. There is even a yearly difference; eggs in the summer of 1941 showed much less response than in 1940 and 1939. Particularly important is the natural variation in sea water concentration along the coast (Garrey, 1915). These variables do not affect the validity of the results, since results are stated in terms of controls run at the same time with eggs from the same material.

From the work of other authors we can conclude that hypotonic stimulation of cell division is not an isolated phenomenon. It speeds up regenerative as well as embryonic processes. Jaques Loeb, in 1892, reported accelerated growth and regeneration of Tubularia hydranths kept in hypotonic sea water (one-third distilled water), and suggested that growth in general decreases with decrease in water content and increases with limited increase in water content. Morgulis (1911) demonstrated that there is actually a high water content in the regenerating tails of the polychaete, Podarke, and the salamander, Triturus. Goldfarb (1907) obtained maximum regeneration with Eudendrium and Pennaria hydranths in sea water diluted to 95 per cent to 80 per cent. Sayles (1928) found that new tissue formation during regeneration in Lumbriculus increased as the medium was diluted below the tonicity of the body fluid. Injecting distilled water into the body cavity (Sayles, 1931) produced the same cellular picture that was found in regenerating worms. Increase in mitoses in the digestive tract was one of the responses to hypotonicity. Aisenberg (1935) obtained increase in mitoses in frog epithelium after immersion in distilled water, or injection of distilled water.

Following the work of Carrel and Burrows (1911), a number of workers verified these authors' finding of an increase in growth of tissue cultured in diluted media. In most cases, however, growth was measured by the area of the outgrowth, and the rôle of mitosis in this increase was not studied. Lambert found no increase in cell division and attributed the increase in area of the cultures entirely to migration of the cells. The experiments of von Möllendorff with fibrocytes of adult rabbits also revealed no acceleration in hypotonic culture media. On the contrary, Živago, Morosov, and Ivanickaja (1934) found that cells of human embryonic heart do proliferate more rapidly in diluted culture medium. Olivio and Gomerato (1932) also found an increase in mitoses in hypotonic tissue culture media. In the first transplant into plasma diluted to half, they found the mitotic index of chick heart was nearly twice that of similar tissue grown in undiluted plasma. In subsequent transplants, the hypotonic cultures from seven-day hearts maintained a higher mitotic index throughout the 11 days of culture (four transplants). However, the mitotic index of the three-day heart dropped below that of the control after the first transplant. Knake (1933) found a selective effect of hypotonicity upon cultures of chick pancreas, resulting in increased growth of epithelium and decreased growth of fibroblasts.

The effects of hypotonicity upon echinoderm development have been reported only for stages later than the initial cleavages. Vernon (1895), working with Strongylocentrotus, and Medes (1917), with Arbacia, obtained larger larvae in slightly diluted sea water. Białaszewicz (1921), however, reports only retardation of Strongylocentrotus and Echinus between the 4 to 12 blastomere stage and the blastula, in all dilutions.

There are some clues as to the mode of action of the hypotonicity. One point at which it takes effect is within the mitotic cycle, inasmuch as cleavage can be accelerated by treatment begun as late as the diaster stage. The greater acceleration obtained when treatment is begun earlier may merely reflect the longer time of action, or may result from a second point of action during the fertilization process. Churney's studies (1940) suggest that the elongation of the egg preparatory to cleavage may be the process sensitive to hypotonic acceleration. He found that elongation of the eggs is proportional to the dilution. If hypotonicity acts at this point, it must do so by allowing the elongation to occur sooner, since merely speeding the elongation once it has begun could not produce accelerations as large as those obtained.

The type of action by which hypotonicity takes effect would seem to be an improvement of the intracellular conditions, bringing them nearer optimum for cleavage, at least. If such is the case, one would expect that each successive division would be equally accelerated. Some of the results reviewed above, particularly those of Olivio and Gomerato, must represent such a sustained effect. Possibly in Arbacia the available energy is quickly exhausted, so that only one cleavage can be hastened. Varying supplies of energy could also explain the large variations between different sets of eggs. However, these experiments are not designed to eliminate the possibility that the acceleration results from a single stimulus rather than a sustained optimum. It is hoped that interrupted dosage, after which the eggs are returned to normal sea water, will reveal whether stimulus or optimum is the accelerating mechanism.

SUMMARY

1. In sea water diluted to a concentration 98 per cent to 94 per cent that of normal sea water, the eggs that responded showed acceleration of the first cleavage

as high as 5.1 per cent of the normal time between insemination and cleavage. Eggs from some sea urchins did not respond, but none showed deceleration in this range of concentrations.

2. In concentrations 92 per cent to 88 per cent, some sets of eggs were accelerated, while others were retarded. This reveals a threshold of antagonism between accelerating and decelerating effects.

3. Concentrations 84 per cent and less always retarded, as did concentrations hypertonic to sea water.

4. Sea water diluted with isotonic electrolyte or nonelectrolyte did not produce an acceleration.

5. The response could be obtained with treatment begun shortly after entrance of the sperm, or as late as the diaster of the first cleavage. The latter indicates that the acceleration results in part, at least, from action upon some phase of mitosis.

6. The second cleavage could be accelerated separately, but eggs left in hypotonic sea water did not show cumulative accelerations for each cleavage. This shows that limited energy is available, or the effect is of the nature of a stimulus.

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THE INDEPENDENT DIFFERENTIATION OF THE SENSORY AREAS OF THE AVIAN INNER EAR

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Introduction

The differentiation of the otocyst of the chick embryo when isolated and transplanted to the chorioallantoic membrane has been studied by Hoadley (1924). Grafts of primordia taken from 48-hour embryos showed membranous labyrinths which, while they were irregular in shape, displayed considerable histogenetic differentiation. This was particularly true of the sensory areas, which presented an histological picture directly comparable to that of the control. As neither nerves nor ganglion cells were found in the grafts Hoadley concluded that in that respect the sensory areas were independent differentiations of the otic epithelium. Fell (1928) studied the development of the 72-hour chick otocyst in tissue culture and obtained labyrinths which showed good histological differentiation but only slight advance in morphogenesis. She reported that the sensory areas of the explanted inner ears were nearly comparable to the sensory areas of the control. No observations on isolated labyrinths which were explanted and treated with a specific nerve stain are recorded by either of these investigators.

The results obtained by Hoadley and by Fell indicate that differentiation of the sensory areas of the chick labyrinth may take place independent of innervation. This cannot be proved, however, until it is demonstrated beyond doubt that there are no nerve fibers present in the immediate vicinity of or associated with well differentiated sensory areas of explanted labyrinths. It is difficult to demonstrate this without resorting to techniques which selectively stain the nerves, many of which are not satisfactory for embryonic preparations. The method of Bodian (1937), however, has yielded excellent results. The following experiments were made in an effort to test the validity of the conclusions of Hoadley and of Fell. The results of the experiments confirm those conclusions.

Material and Methods

In the following experiments grafts to the chorioallantoic membrane were made essentially as described by Hoadley. Chick embryos of ages ranging from 12-somites to 43-somites were placed in warm mammalian Ringer's solution on a warm stage on the stage of a binocular dissecting microscope. Fine knives made from dissecting needles were used in the operations which were performed by aid of a low magnification of the microscope. The otic region was removed and treated in either of two fashions. Some of the transplants were composed of the otocyst together with the surrounding mesenchyme and a portion of the adjacent myelencephalon; in other cases the otocyst was freed and then cleaned of as much of the adhering mesenchyme tissue as was possible in view of the haste required in the operation and the desire to avoid mechanical injury to the primordium. In most of the later transplantations, the otocyst alone was wrapped in a mantle of somatopleure taken from the lateral plate region of the area pellucida. Such treatment materially increased the percentage of successful grafts, apparently by protecting the donor tissues in the interval before complete incorporation by the host membrane. An envelope of splanchnopleure was also tested in the transplants but the somatopleure proved to be superior for the purpose. Control transplants of both somatopleure and splanchnopleure alone as well as of mesenchyme from the otic region were made and examined.

The grafts were fixed in Allen's P.F.A.$_3$ fixative, sectioned at 10 μ and stained with activated protargol according to the method of Bodian (1937).

Experimental Results

Transplants of inner ear rudiments isolated from embryos of several different ages have been made and numerous grafts have been recovered. Identification of parts of these graft ears has been made largely on the basis of histological differentiation, but in some instances, the morphology of the labyrinth has been used to supplement the histological picture.

Since many of the younger otocysts were wrapped in either somatopleure or splanchnopleure from the lateral plate region before transplantation and all explants had some mesenchyme cells clinging to them, it was thought well to determine what embryonic structures might arise from these tissues when transplanted to a foreign environment. To do this, some transplants were made of somatopleure alone, some of splanchnopleure, and some of the mesenchyme adjacent to the otocyst. Graft A184–41 is of somatopleure taken from the lateral plate region of a 31-somite donor and has a control age of 9½ days. There is no differentiation of embryonic structures in the graft. The same result was obtained when splanchnopleure of the lateral plate region of a 30-somite (ca.) donor was grown for 7 days.

Considerable differentiation is found in some grafts of mesenchyme taken from the region of the otocyst. Two cases are worthy of mention, because ganglion cells and cartilage are present in both. Unfortunately, the exact region from which the mesenchyme was taken is not recorded in the protocols. Case A192–41 is of mesenchyme taken from a 31-somite donor and has a control age of 9½ days. It contains an elongated nodule of well-differentiated cartilage and beyond one end of this cartilage is a ganglion. Case A182–41 is of mesenchyme from a 28-somite donor. It has a control age of 9 days. A cartilage nodule is present with ganglion cells localized at one side of it. There are several epithelial pearls in this graft.

The presence of cartilage in the grafts of mesenchyme indicates that by 28 somites the mesenchyme in the region of the otocyst possesses the capacity to form cartilage even though it is isolated from the influence of the membranous labyrinth. The ganglion cells which are present in both of these grafts probably mean that some cranial neural crest material or some cells from the field of the acoustico-facialis ganglion were transplanted with the mesenchyme.

Since the grafts recovered are too numerous for complete description, they have been divided into convenient groups based on the stage implanted and typical examples from each group will be fully described. Information secured from some of the other grafts which will not be described is presented in Table I.

It is extremely difficult to determine the specific identity of a sensory area in a graft. Because of this, thickenings of the otic epithelium which tend to resemble cristae are referred to as cristae and the macula-like sensory areas are designated as maculae. It should be borne in mind that these terms are used in a descriptive sense and do not always imply a positive identification of a specific sensory area.

TABLE I

Data on some of the grafts of the otic region which are not described in the text

Case number	Donor age	Control age in days	Remarks
A2–40	17S	9½	Several sensory thickenings and most of them are innervated.
A247–41	18S	8½	Two otic pits and brain implanted. Both inner ears have differentiated sensory areas. Some of these are innervated.
A327–41	25S	8¾	Good morphological differentiation. No ganglion cells in graft and no differentiation of sensory areas.
A140–41	26S	8	Two sensory areas with no nerves to their epithelia.
A244–41	28S	9	Nerves and ganglion cells present but no differentiation of sensory areas.
A169–41	31S	8½	Shows utriculus, sacculus, canal rudiment and endolymphatic evagination. Half-moon of cartilage around labyrinth. No nerves in graft. Contains a well differentiated crista and a macula.

12–20 Somites. Case A299–41 is a 7-day graft of an otic placode isolated from a 12-somite donor. Before transplantation the placode was wrapped in somatopleure. The membranous labyrinth consists of two vesicular structures of unequal size connected by a small lumen and has an endolymphatic duct and sac. There is no development of sensory areas in the epithelium of the smaller vesicle, but the larger vesicle has several sensory areas. The high columnar epithelium of the endolymphatic sac is more convoluted than in the control. No canal rudiments are present. A rod-shaped cartilage mass is found along one side of the ear. There are a few ganglion cells and short nerve processes between lobular outpocketings of the endolymphatic sac but these neurones are localized and no nerves approach the sensory epithelium. Three cristae which appear to be differentiations of a large patch of thickened epithelium are present in the larger vesicle. There is no definite development of hair and supporting cells, but some short dark-staining cells which reach the surface of the epithelium suggest the initial differentiation of hair cells. No nerves enter the sensory areas. These sensory areas do not project as far into the lumen as the cristae of the control.

Near one end of the larger vesicle there are two small, flattened epithelial thickenings with neither otolithic membranes nor differentiated hair cells. Their form is similar to that of a macula but the small size and lack of differentiation make their identity somewhat problematical.

Another graft (A302–41) in this group is of an otic pit isolated from an 18-somite embryo. This was not wrapped in either somatopleure or splanchnopleure before implantation. The control age is $8\frac{1}{2}$ days. This inner ear is vesicular and has an endolymphatic duct and sac. Well differentiated cartilage forms a half-moon around the side of the labyrinth which is away from the endolymphatic duct (Figure 1). Ganglion cells and nerves are present in the graft but they are not found in the immediate vicinity of the sensory epithelium. A crista (Figures 1 and 2) has a slight indication of a covering membrane but hair and supporting cells are not clearly differentiated. No nerve elements can be distinguished among the connective tissue cells which underlie the sensory epithelium.

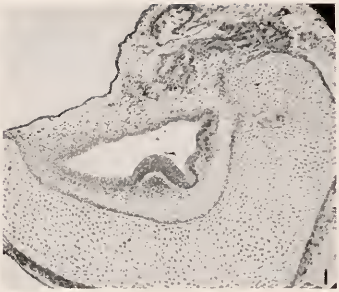
The nerve fibers in this graft originate from ganglion cells located in the mesenchyme to one side of the otic epithelium. From these cell bodies the fibers pass through a break in the cartilage to a ganglion located beside the endolymphatic sac. Some nerves from this latter ganglion pass close to the epithelium of the endolymphatic sac and are lost in the surrounding connective tissue. Another branch continues through the break in the cartilage and passes in the opposite direction along the outer edge of the cartilage to a brain-like mass of nervous tissue. The presence of nerve elements indicates an incomplete isolation of the otic pit. At this stage it is difficult to clean the ear rudiment before transplantation. A significant feature of both these labyrinths is the complete separation of definite sensory areas from nerve fibers and ganglion cells.

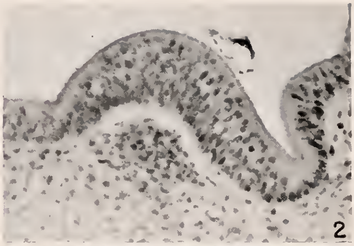
21–25 Somites. The grafts in this age group show more complete morphological differentiation than do those described in the preceding section (cf. Waterman and Evans, 1940). Correlated with this is an increase in the completeness of histogenesis.

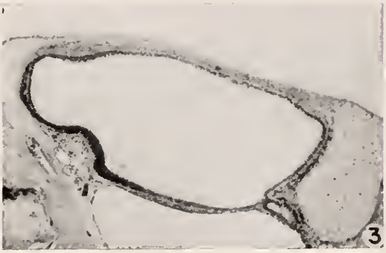
The most extensively developed graft, A397–41, is of an otic pit isolated from a 21-somite donor. Its control age is $8\frac{3}{4}$ days. A utriculus, a sacculus and two canal rudiments are present. A portion of the saccular region has been interpreted as a lagena, because of the similarity of its sensory epithelium to a papilla basilaris. There is no indication of development of the recessus labyrinthi. This is unusual, for this part is one which persists in practically all grafts of the inner ear. The otic capsule is represented by three nodules of cartilage.

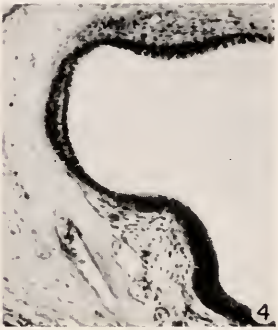
There are four foci of thickened epithelium which represent the sensory areas but none of these sensory areas is innervated. A crista is found near the utricular end of a canal rudiment and the other canal rudiment has a macula near its base. This latter sensory area is in a small pocket between the utricular ends of the two canal rudiments. The sensory area of the lagena has some clear cells to one side which are similar to those of a papilla basilaris. There is differentiation of hair and supporting cells, but the membrane which covers this sensory area resembles an otolithic rather than a typical tectorial membrane. The rest of the thickened epithelium of this lagenar sensory area resembles a macula but has no differentiation of hair or supporting cells.

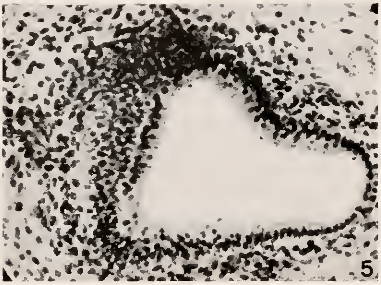
Beyond and to one side of the blind ends of the canal rudiments are three groups of nerve cell bodies. There are 2–4 cells per group and capsule cells are evident at the periphery of these cell bodies. A few nerve fibers run toward these groups of cells from the region of the otic epithelium but the fibers are not stained heavily enough in the vicinity of the cell body to trace them into the cell itself. In the other direction, these fibers disappear in the mesenchyme. A second region of ganglion cells lies between the epithelium of the lagena and the edge of the graft. The most prominent of these cells is a bipolar neurone lying close to the











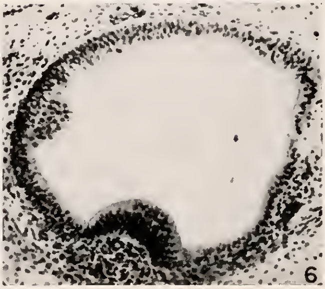


PLATE I

1. Section through graft A302–41. The endolymphatic sac is represented by convoluted epithelium near the top of the photograph. Two oval areas in the cartilage at the right mark the nerves which pass through the cartilaginous capsule. A well formed crista is seen on the lower side of the lumen. × 45.

2. Higher power view of the crista of Figure 1. × 195.

3. Section through graft A120–41. The macula is at the extreme left of the photograph. A crista is present slightly to the right and below the macula. × 60.

4. Higher power view of the macula and crista seen in figure 3. × 112.

5. Section through the innervated sensory area of graft A163–41. Nuclei of hair cells may be seen near the surface of the macula which is on the left of the lumen. Nerve fibers in the area underlying the macula appear as faint lines in the figure. × 178.

6. Section through graft A163–41 at another level. A crista is present at the lower margin of the lumen. The mass of cells at the left which projects into the lumen represents the termination of the innervated sensory area seen in Figure 5. × 110.

lagenar epithelium. One process of this neurone bends back on itself and joins other nerve fibers which run through the thickened sensory epithelium of the sacculus just inside and parallel to the basement membrane. They soon leave the sensory epithelium and pass along the closed end of the lagena into the mesenchyme.

Case A151–41 is of an inner ear rudiment which was isolated from a 24-somite donor and wrapped in somatopleure before transplantation. Its control age is 9 days. The labyrinth is somewhat vesicular but histologically a utriculus, sacculus and an endolymphatic duct and sac are distinguishable. There are no canal rudiments. The cartilage capsule is confined to the utricular end of the ear. It surrounds the utriculus at its distal end but forms only a nodule at one side of the proximal region. Poor development of the cartilage capsule is taken as evidence that little adjacent mesenchyme was transplanted with the otic rudiment.

A crista is found in the utriculus and a macula is present in the sacculus. The crista shows no evidence of subordinate differentiation or of innervation. There are some ganglion cells outside of the cartilage capsule. From these cells, nerves pass through a break in the capsule, run beneath the macula and end in the adjacent mesenchyme. Just beneath the free surface of this macula and parallel to it are some fibers which stain black with Bodian's technique. Although their cell bodies are not evident, these are interpreted as nerves and the sensory area is probably innervated by these fibers. The innervation is not typical. There is some suggestion of hair and supporting cells in this macular area and the epithelium is thrown into shallow humps at two places. A poorly developed otolithic membrane is present.

27–30 Somites. Operations performed on embryos of these ages are easily accomplished by shelling out the otocyst, but it is difficult to free the inner ear rudiment from all of the ganglion cells. The exact relation of otocyst and ganglion cells is indefinite in control embryos and the number of grafts in this group which showed nerve cell bodies and nerves connecting with the otic epithelium make it reasonably certain that the nervous elements were not successfully excluded in the majority of cases.

Case A336–41 is a $6\frac{1}{2}$ day graft of an otocyst isolated from a 27-somite embryo. Utriculus, sacculus, endolymphatic sac and duct, lagena and three canal rudiments can be distinguished. The cartilage capsule is almost complete, especially around the lagena and sacculus. The endolymphatic sac and duct are inside the cartilage capsule next to the otic epithelium instead of being external to the capsule as in the control. In all, five sensory areas are represented.

Two cristae and one macula are found in the utriculus. The cristae are on opposite sides of the utriculus with the macula beside one of the cristae. Both of the cristae are innervated and show slight differentiation of hair and supporting cells. The presence of cupular material is questionable. The macula is poorly differentiated with no clearly defined hair or supporting cells and no innervation.

The sacculus is a bilobed sac-like structure which joins the utriculus opposite the point of entry of the endolymphatic duct. It has a macula which is innervated and shows the initial differentiation of both hair and supporting cells. The lagena appears as a direct continuation of the utriculus and contains a fairly well differentiated sensory area which is similar to a papilla basilaris. Beside the lagena there is a large ganglion from which nerve fibers run to the cristae and to the sensory areas in the sacculus and in the lagena. A few nerve cell bodies are found beside the sacculus and some more are adjacent to the lagena.

A second graft of an otocyst from a 27-somite donor (A120–41) has only one of its three sensory areas innervated. This otocyst was wrapped in splanchnopleure before implantation and has a control age of 8¼ days. That this was a more successful isolation than case A336–41 is suggested by the fact that the cartilage capsule is less complete than in the previous case. The cartilage forms a half-moon around the labyrinth except at one extremity where it assumes an elongate rod-shaped form (Figure 3). A utriculus, sacculus, endolymphatic appendage and two canal rudiments may be distinguished.

Two cristae and a macula are found in the utriculus. The cristae are on opposite sides of the utriculus and are not innervated. They show no differentiation of hair and supporting cells. The macula is located near one of the cristae and has both hair and supporting cells (Figures 3 and 4). On opposite sides of the macula are two sets of nerve cell bodies which are connected by nerves. These nerves pass through the sensory epithelium and it is probable that they give off some branches which innervate this sensory area although the innervation is not typical.

The third graft to be described (A238–41) is of an otocyst isolated from a 30-somite donor. Its control age is 9½ days. Utriculus, sacculus, lagena, endolymphatic duct and sac and one canal rudiment are represented. The cartilage capsule forms a half-moon around the labyrinth. A non-innervated crista is present in the utriculus and the sacculus contains extensive sensory epithelium of the macular type. Adjacent to the sacculus is a ganglion containing the cell bodies of several nerves which run beside the macula but enter its epithelium at only a few points. The macula is covered by an otolithic membrane. The lagena is identified by some clear columnar cells which are typical of the papilla basilaris but this papilla basilaris is poorly differentiated and is not innervated.

31–43 Somites. In transplants of older otocysts, there tend to be more well-differentiated sensory areas than were present in grafts of younger stages. A striking thing about this present group is the relatively few sensory areas which show signs of innervation. In general, morphogenesis has proceeded further in these transplants than in transplants of younger otocysts, although vesicular inner ears are found in the grafts.

Case A163–41 is an otocyst isolated from a 32-somite donor and wrapped in somatopleure before implantation. Its control age is 8½ days. This inner ear is an ovoid vesicle with an endolymphatic duct and sac entering from one side. There are no canal rudiments nor is there any epithelial differentiation of the pars inferior labyrinthi. At one end of the labyrinth the cartilage capsule completely surrounds the otic epithelium but the rest of the labyrinth has only a half-moon shaped capsule around the side opposite to the endolymphatic duct and sac.

There are three general regions of thickened epithelium which represent four sensory areas. Two of them are well defined, but the third is spreading and is composed of a crista and a macula. At least a part of all of these sensory areas is apparent in the portion of the otic epithelium which is surrounded by cartilage, and two of them extend into the region of the labyrinth which has only a half-moon of cartilage around it. The macular portion of the large spreading area of sensory epithelium begins in the portion of the labyrinth where the capsule is complete. Here the sensory epithelium is stratified and has a suggestion of a covering membrane. The sensory area has a flattened macula-like appearance with both hair and supporting cells present. The macula (Figure 5) is innervated by nerve fibers which pass through a break in the cartilage capsule; the cell bodies of these nerves were not identified. The sensory epithelium soon increases in area and changes from a flattened to a hillock-like form which is interpreted as a crista. It has both hair and supporting cells as well as a cupular remnant. This sensory epithelium terminates just before the endolymphatic duct enters the vesicular portion of the labyrinth. In the region where the complete cartilage capsule becomes half-moon shaped, there is a thickening of the otic epithelium into a crista-like sensory area (Figure 6). There is evidence of both hair and supporting cells, although the presence of sensory hairs is questionable. The smallest sensory area is a thickening of the epithelium in which stratified cells form a small crista. This is not well differentiated, for neither hair nor supporting cells can be distinguished and cupular material is lacking. This labyrinth is noteworthy because of the large areas of well differentiated sensory epithelium which are not innervated.

A graft of an otocyst from a 43-somite donor (A382–41) shows more morphological differentiation than does the preceding case. This otocyst had the endolymphatic rudiment cut off prior to implantation. The control age is 12 days. A utriculus, sacculus, lagena, two canal rudiments and a complete canal are present. The sensory areas are well differentiated. The cartilage capsule surrounds the portion of the utriculus which communicates with the complete semicircular canal but in other places it is made up of several cartilage nodules which differ in size.

Seven thickenings of the epithelium probably represent sensory areas. Four of them are widely separated from each other, but the other three are on the same side of the labyrinth and are so close together that they may well have arisen by a splitting of a single area of sensory epithelium. In the pars inferior portion of the inner ear are found a macula as well as some neuroepithelium which resembles a papilla basilaris. The macula has stratified epithelium, is flattened and probably represents the macula sacculi. Supporting cells and hair cells with sensory hairs which project above the surface of the macula are evident. The macula is covered by an otolithic membrane. The sensory area of the lagena is identified by its flattened shape and stratified epithelium surmounted by a tectorial membrane. This membrane has been separated from its attachment point but its position is similar to the tectorial membrane of the control. Well defined hair and supporting cells are present. The hair cells are close to the surface as in the papilla basilaris of the control and sensory hairs project above the epithelium.

The utriculus contains five sensory areas. The one which is close to the saccular region is flattened and shows both hair and supporting cells. Sensory hairs and an otolithic membrane are present. This sensory area is interpreted as a macula utriculi. Almost in continuity with this sensory area is another flattened region of well differentiated neuroepithelium which resembles the macula described above. The macula is continuous with a well differentiated crista which occurs at the entrance of the complete canal. The histological picture suggests an ampulla with its crista. Above the crista is a covering which may represent cupular material but it does not resemble a typical cupula. A sixth area of neuroepithelium is found at the base of a canal rudiment. It has a typical crista shape and is well differentiated. There are both hair and supporting cells as well as sensory hairs. As in the case of the other crista in this graft, there is some dark-staining material above the epithelium which is interpreted as a cupular remnant. The seventh sensory area is a crista at the opposite side of the utriculus from the two well differentiated cristae which are described above. Hair and supporting cells are not well differentiated and the presence of sensory hairs is questionable. There are no nerves in this graft.

An interesting feature of this group of transplants is the absence of nerves in a large proportion of the grafts. In two cases (A377–41, A163–41) there is only one innervated sensory area in each labyrinth and case A187–41 has three innervated sensory areas which are near each other. Three other grafts examined in this group had no nerve elements present.

The differentiation of the components of the ear was also tested by transplanting portions of the otocyst from donors of this age group. Two grafts (A375–41, A380–41) of the endolymphatic rudiments of otocysts from 43-somite donors show that this portion may survive and grow in a graft. The epithelium is convoluted and the columnar cells are much taller than in the endolymphatic sac of the 12-day control. Case A382–41, previously described in this group, had the endolymphatic rudiment removed before implantation. This labyrinth showed no evidence of an endolymphatic duct or sac. Apparently, the inner ear cannot regenerate this portion of itself in a graft when the endolymphatic rudiment is removed at the 43-somite stage.

Two grafts of the isolated pars inferior were recovered. Case A369–41 is a graft of the pars inferior of an otocyst from a 41–somite donor. Its control age is 12 days. This labyrinth consists of two vesicular structures. A part of one of the vesicles has differentiated as a lagena and shows a well developed papilla basilaris. No other special epithelial differentiations were observed. The other graft (A347–41) is not as completely differentiated as this one. It is of a pars inferior from a 38-somite donor and has a control age of 10½ days. A lagena and sacculus are present but the only suggestion of a sensory area is some stratified epithelium which shows no specific differentiation of hair and supporting cells. These cases indicate that under the conditions of the experiment the pars inferior is incapable of regenerating a pars superior and recessus labyrinthi by the stage at which these transplants were made.

Discussion

The transplanted labyrinths which have been studied show that cristae, maculae and a papilla basilaris will differentiate in grafts of the inner ear rudiment, although these sensory areas may not be innervated. An isolated labyrinth may have more than one of these types of sensory areas, but it has been found that generally a papilla basilaris does not occur in a graft when no other type of sensory area is present. For the most part, these sensory areas are well developed and, while not always comparable to the control in all respects, their differentiation indicates that histogenesis has been only slightly retarded. The completeness of differentiation of individual sensory areas is not affected by the age of the donor, although the otocysts which are older at the time of transplantation contain more numerous sensory areas than do otocysts from younger donors. The sensory areas may or may not be innervated.

The results reported here are in accord with those of Hoadley (1924) and of Fell (1928). Their studies and the present results indicate that the sensory areas of the inner ear of the chick possess a considerable capacity for independent differentiation. When the nerves are prevented from reaching the otic epithelium, the histological differentiation of the sensory areas is retarded only slightly if at all. In connection with this study, some transplants were made which included the myelencephalon and the otocyst in their usual relations. These labyrinths do not show innervation of all of the sensory areas. At least one sensory area in each labyrinth has no nerve fibers running into it. The cases where nerves penetrate the epithelium do not yield any more highly differentiated sensory areas than do those grafts of isolated otocysts in which there are no nerves in the vicinity of the sensory epithelium. Other sense organs beside the sensory areas of the inner ear are capable of differentiating when isolated from the influence of the nervous system. The studies of Harrison (1904) on the lateral line organs of anurans show that these organs can develop and differentiate in the absence of nerve fibers. Several more recent investigators have examined the capacity for independent differentiation possessed by taste buds in a number of forms.

The results reported here present strong evidence that the sensory areas of the avian inner ear are capable of independent differentiation from the otic epithelium after the 12-somite stage, at least in so far as the nervous system is concerned. Since in otocysts isolated from nerve elements, sensory areas appear which are histologically comparable to those of the control, the nerves themselves appear to play no part in the development of the sensory areas and may be only passive elements which are attached to the sensory epithelium and follow its divisions during the development of the sensory areas. If the nervous system exerts any "initiating" influence upon the otic epithelium which results in the formation of sensory areas, this effect probably occurs prior to the stages studied here. That would place the time of action previous to the appearance of the first demonstrable nerve fibers in either the central or the peripheral nervous system.

SUMMARY

The capacity for differentiation of the sensory areas of the avian inner ear independent of innervation, has been studied by transplanting isolated primordia of the inner ear to the chorioallantoic membrane. Maculae, cristae and a papilla basilaris differentiated in the transplants. All three types of sensory areas are seldom found in any one graft. The sensory areas of the transplanted labyrinths are comparable to those of the control. The morphogenesis of the membranous labyrinth was greatly suppressed in the grafts but the histogenesis of the sensory components showed but little retardation.

Since the sensory areas of the inner ear undergo typical development when isolated from their nerve supply, it is concluded that they are capable of independent differentiation in so far as the nervous system is concerned.

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THE UTILIZATION OF GLYCOGEN BY FLIES DURING FLIGHT AND SOME ASPECTS OF THE PHYSIOLOGICAL AGEING OF DROSOPHILA

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The frequencies with which many insects move their wings during flight are unparalleled in the appendicular movements of any other animals. The extent to which this is true may be judged from Table I where the maximum frequencies of muscular movements yet recorded for reptiles, birds, and mammals are compared with the frequency of wing-beat of Drosophila. The intense level of activity characteristic of flight is also revealed by the high rates of oxygen consumption characteristic of flying insects (Chadwick and Gilmour, 1940; Davis and Fraenkel, 1940; Krogh, 1941). For these reasons the metabolic processes responsible for flight are of unique physiological interest.

TABLE I

Wing-beat frequency of Drosophila and the maximum frequencies yet recorded for the muscular movements of other animals.

Animal	Activity	Frequency (cycles/sec.)	Authority
Drosophila	Wing-beat during flight	100–300	Chadwick & Williams (unpublished data)
Rattlesnake	Movement of rattle	17–100	Chadwick & Rahn (unpublished data)
Humming-bird	Wing-beat during flight	60–70	Edgerton & Killian (1939)
Mouse	Scratching reflex of hind leg	20	Chadwick & Pearson (unpublished data)
Man	Voluntary vibration of the *opponens pollicis* muscle of the hand	10–13	Schäfer, Canney & Tunstall (1886); see also Fenn (1932)

It is known from the studies of Beutler (1936a and b; 1937) that the high concentration of sugar in the blood is utilized by the honey-bee during flight. Glycogen in this animal apparently plays only a minor role in metabolism. However, the dependency of the honey-bee on the food reserves of the hive renders it atypical. This fact along with the high concentration of glycogen characteristic of many other insects (Babers, 1941) suggests that glycogen may be of more general importance in the physiology of flight.

We have sought in the present investigation to test this possibility by studying the flight metabolism of flies. By combining microchemical analyses for glycogen with stroboscopic determinations of the frequency of wing movement, it has been possible to ascertain the changes in the concentration of glycogen which accompany measured amounts of flight activity.

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MATERIALS AND METHODS

The study was performed, for the most part, on a strain of *Drosophila funebris* (Fabr.) that had been previously inbred brother by sister for ten generations. Female individuals were used exclusively. Age was controlled within ±2 hours during the first two days of adult life and within ±12 hours in older individuals. The flies were raised and isolated in bottles containing a standard agar-molasses-yeast culture medium in a room having a constant temperature of 20.0 ± 0.5° C. Female individuals of an inbred strain of the blow-fly, *Lucilia sericata* (Meig.), were used in one series of experiments.

Measurements of the frequency of wing-beat were carried out by means of an Edgerton stroboscope. This instrument consists of a neon-filled tube whose flash-frequency can be varied by means of a potentiometer. When the flash-frequency is tuned to equal the frequency of wing movement, a standing-image of the wings is obtained and the calibrated scale of the stroboscope then indicates the frequency of the wing-beat.

The measurements were performed on animals during "fixed" flight. This was accomplished by attaching the posterior, dorsal tip of the abdomen by means of paraffin to a wire which served as a support. Drosophila, after the initiation of flight, generally flew until exhausted, whereas it was necessary to stimulate Lucilia continuously by means of a slight movement of air produced by an electric fan. Lucilia was flown at room temperature and Drosophila at 20.0 ± 0.5° C.; stroboscopic measurements of wing-beat frequency were made on each individual every 3 minutes or more often.

Glycogen was determined by the Pflüger method with modifications by Good *et al* (1933) and Blatherwick *et al* (1935). After acid hydrolysis of the glycogen, the concentration of the resulting glucose was measured by the copper-iodometric method of Shaffer and Somogyi (1933) using Reagent 50. The analytical process was calibrated by means of C.P. glycogen and glucose.

UTILIZATION OF GLYCOGEN DURING FLIGHT

A group of 4- to 5-day old Drosophila, which had been isolated in a single bottle of food, were mounted and stimulated to fly. Each half hour a number of animals were stopped and immediately analyzed *in toto* for glycogen. The results are recorded in Table II and Figure 1. The glycogen was found progressively to

TABLE II

The utilization of glycogen during the continuous flight of Drosophila funebris.

Duration of flight (minutes)	Number of animals	Mean concentration of glycogen (in per cent of live weight)
0	12	4.88
30	10	3.93
60	9	2.73
90	7	1.30
	38	

diminish during flight. At the end of 90 minutes the concentration had decreased from an initial value of 4.88 to 1.30 per cent of the live weight. An extrapolation of the curve in Figure 1 indicates that the concentration of glycogen would reach zero by about the 110th minute of flight. This is in good agreement with the average length of time 4.5-day old Drosophila can fly, which, as can be seen from Figure 4, amounts to 106 minutes.

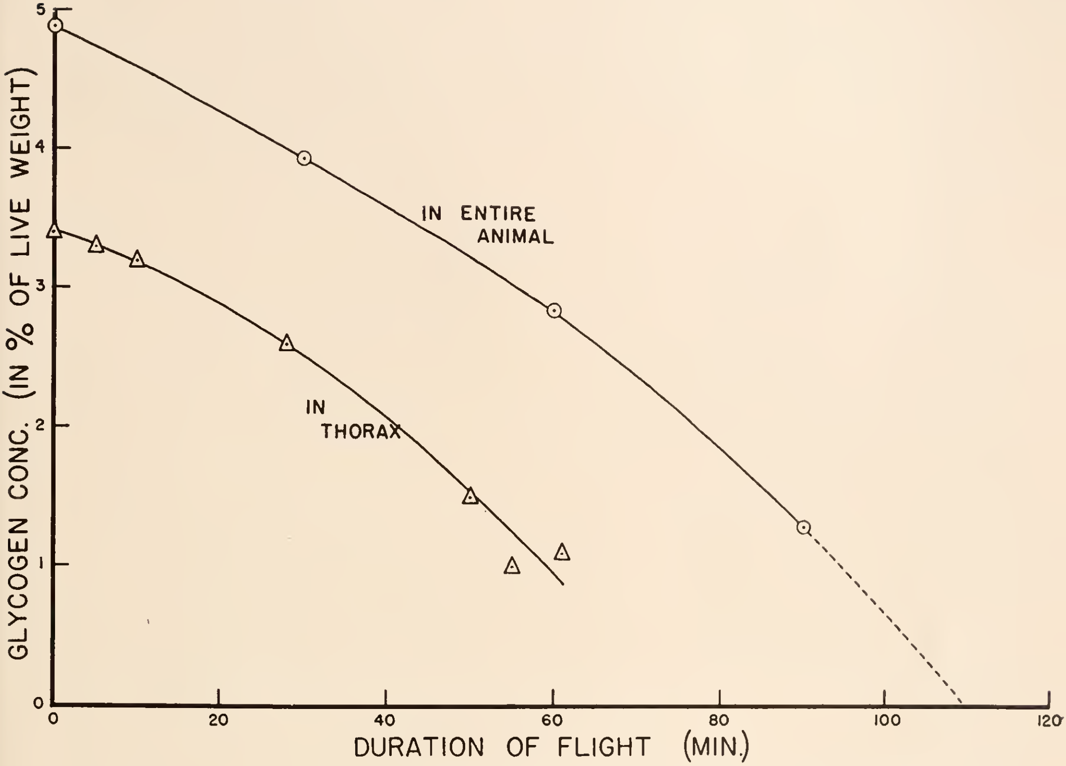

FIGURE 1. Changes in the glycogen concentration of flies during continuous flight. The upper curve was obtained from analyses of entire Drosophila, the lower curve from analyses of the thoraces of Lucilia.

In order to ascertain whether the concentration of glycogen decreases in the thorax during continuous flight, the experiment was performed using Lucilia.

TABLE III

The decrease in thoracic glycogen during the continuous flight of Lucilia sericata.

Duration of flight (minutes)	Number of animals	Mean concentration of glycogen (in per cent of wet weight of thorax)
0	7	3.4
5	1	3.3
10	2	3.2
28	1	2.6
50	1	1.5
55	1	1.0
61	1	1.1

As shown in Table III and Figure 1, the results obtained from the analyses of individual thoraces were essentially identical with those obtained from entire Drosophila.

Wing-beat Frequency During Continuous Flight

The changes which occur in the frequency of wing-beat during continuous flights to exhaustion reflect the response of the neuromuscular system, in terms of the frequency of its activity, to the progressive decrease in glycogen concentration.

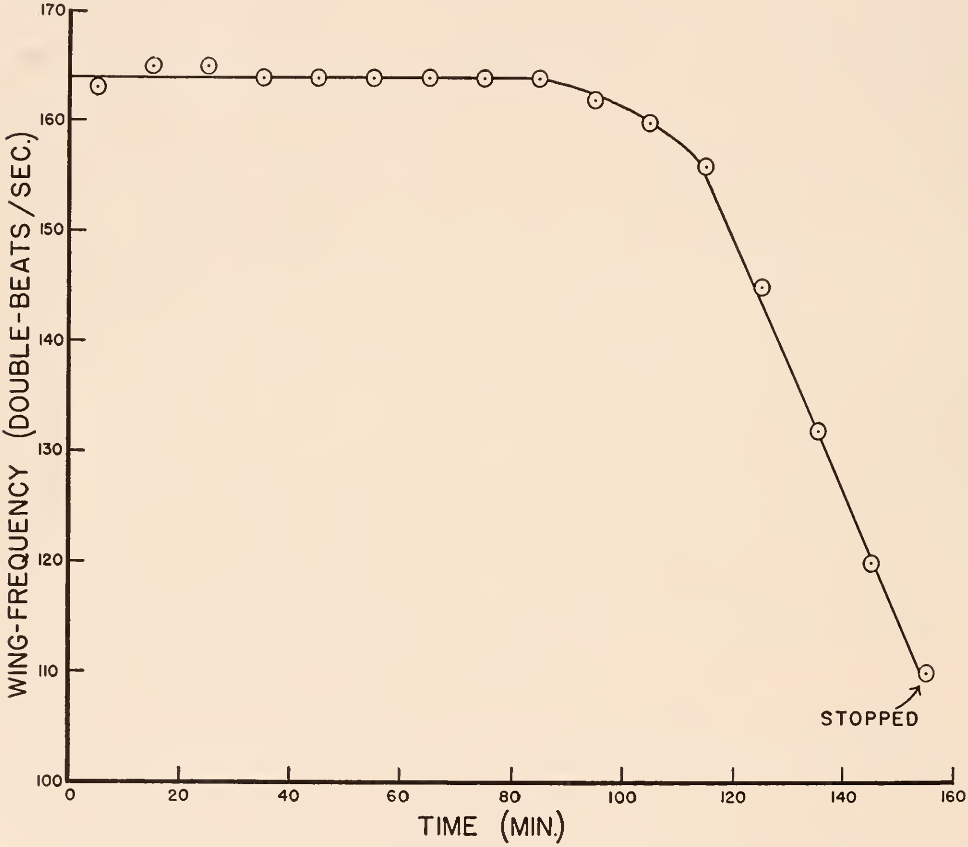

Figure 2. The wing-beat frequency of Drosophila during a continuous flight to exhaustion. The frequencies of wing movement were measured at 10-second intervals and averaged over 10-minute intervals. Animal five days old.

The exact shape of the fatigue curve obtained when wing-beat frequency is thus considered as a function of time differs in detail among the various species of Drosophila and among individuals of a single species. For *Drosophila funebris* it was nevertheless clear that, except in very young or very old individuals, the frequency of wing-beat did not ordinarily undergo any large change, at 20° C., during a considerable period after the initiation of flight. This is apparent in the typical flight shown in Figure 2. Thus the response may generally be divided into two stages: (1) an initial period during which wing-beat frequency undergoes only slight variations; and (2) a final period of fatigue, manifested by a rapid decrease in wing-beat frequency until the termination of flight.

If an animal that has been flown to exhaustion is again stimulated to fly, wing-beat frequency rises momentarily, but then decreases rapidly to the low frequencies characteristic of the final minutes of the preceding flight. Such repeated flights after exhaustion are always of short duration even if the animal is permitted to rest for several hours.

It is noteworthy that the frequency of wing-beat during the final period of fatigue never decreases gradually to zero: wing movement ceases before the frequency becomes as low as 100 double-beats per second. Under no combination of environmental conditions yet tested has any species of Drosophila been induced to fly during "fixed" flight at frequencies lower than about 70 double-beats per second. This fact may eventually be of considerable interest in interpreting the physiology of the neuromuscular system responsible for flight, since it indicates that the flight mechanism not only operates at unparalleled frequencies, but, in the case of flies, is incapable of slowing down to the range of frequencies characteristic of the neuromuscular systems of animals other than insects (see Table I).

Changes in Glycogen Content and in Flight Ability as Functions of Age

Since systematic changes in glycogen content have been reported as functions of age in both insects (Babers, 1941) and mammals (Heymann and Modic, 1939), such variations if found for Drosophila would offer an opportunity of testing for simultaneous changes in flight ability. For this reason the glycogen concentration and the flight ability of Drosophila were studied over the first month of adult life.

Considerable variation was found for these factors among animals of similar age which had been isolated in different bottles of food; in contrast, individuals isolated in a single bottle showed a much higher degree of uniformity. This variability found among animals of similar age may, in part, be attributed to the lack of environmental uniformity within different bottles. For instance, even though the animals were transferred to fresh food every few days, it was impossible to control the yeast growth and the ventilation and, hence, the tensions of carbon dioxide and alcohol vapor. For these reasons it was necessary to use a large num-

Table IV

Changes in the glycogen concentration in Drosophila funebris *as a function of adult age.*

Average age (days)	Number of animals	Average concentration of glycogen (in per cent of live weight)
0.5	91	2.4
3	72	5.1
5	114	6.0
7	65	6.2
10	77	6.2
14	85	6.5
17	75	5.6
19	31	5.3
21	21	4.3
33	23	3.5

ber of determinations of glycogen and of flight ability in order to establish the approximate values for the age relationships.

Fifty-one analyses were performed on a total of 654 animals of known ages. The results are recorded in Table IV and Figure 3. The concentration of glycogen, in terms of its percentage of the live weight, was found to increase during the first two weeks until about 6.5 per cent of the animal consists of this substance. Thereupon, the concentration decreases rapidly and then more slowly during the remainder of the month. The relationship is not significantly altered if the glycogen concentration is computed in terms of dry weight. Since water makes up 60 to 72 per cent of the live weight of the animal, the concentration of glycogen thus increases during the first two weeks until it accounts for nearly one-fifth of the insect's dry weight.

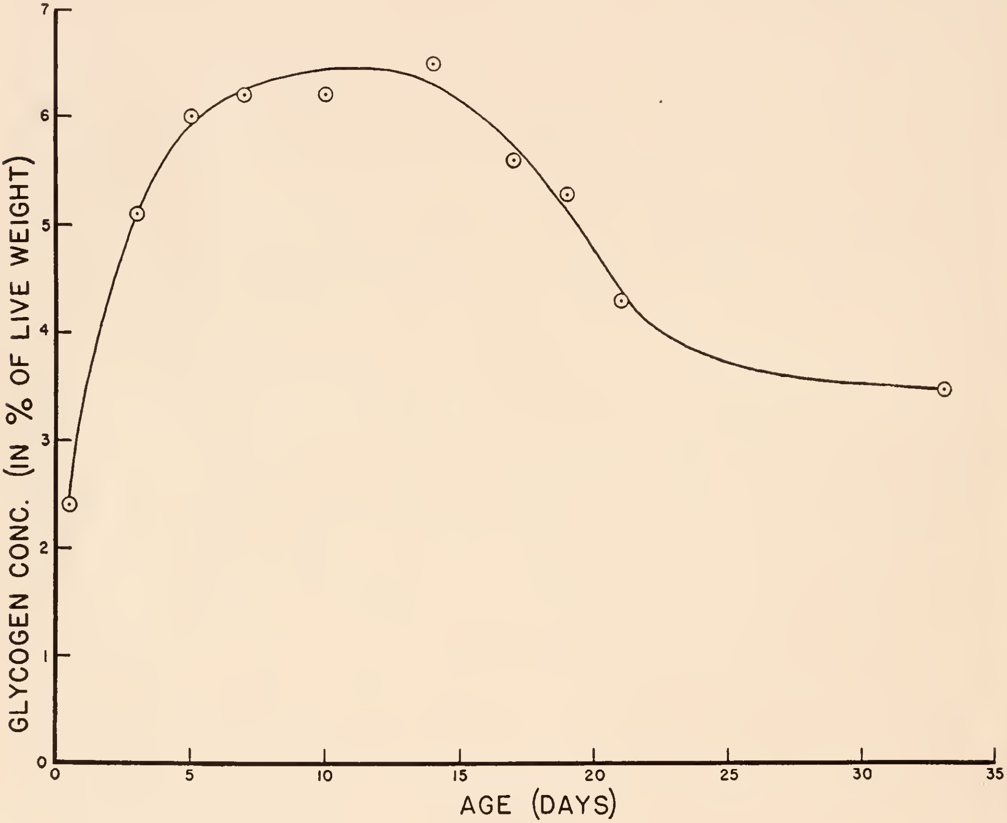

FIGURE 3. Changes in the glycogen concentration of Drosophila as a function of the animals' adult age.

The degree to which flight ability also varies with age was studied on a total of 117 individuals, only small samples being used from each food bottle. Since the wing-beat frequency of each animal was measured at frequent intervals during its continuous flight to exhaustion, the flight ability can be interpreted in terms of the total number of wing-beats as well as in terms of the duration of flight (Table V and Figure 4).

TABLE V

The relation between adult age and the flight ability of Drosophila funebris *in terms of the average duration of flight and the average total number of wing-beats in flights to exhaustion at 20.0 ± 0.5° C.*

Average age (days)	Number of animals	Average duration of flight (minutes)	Average total number of wing-beats
1	18	25.8	225,000
2.5	15	97.7	809,000
6.5	26	110.0	1,022,000
14.5	20	102.3	910,000
18.5	23	38.7	331,000
33.5	15	19.0	172,000

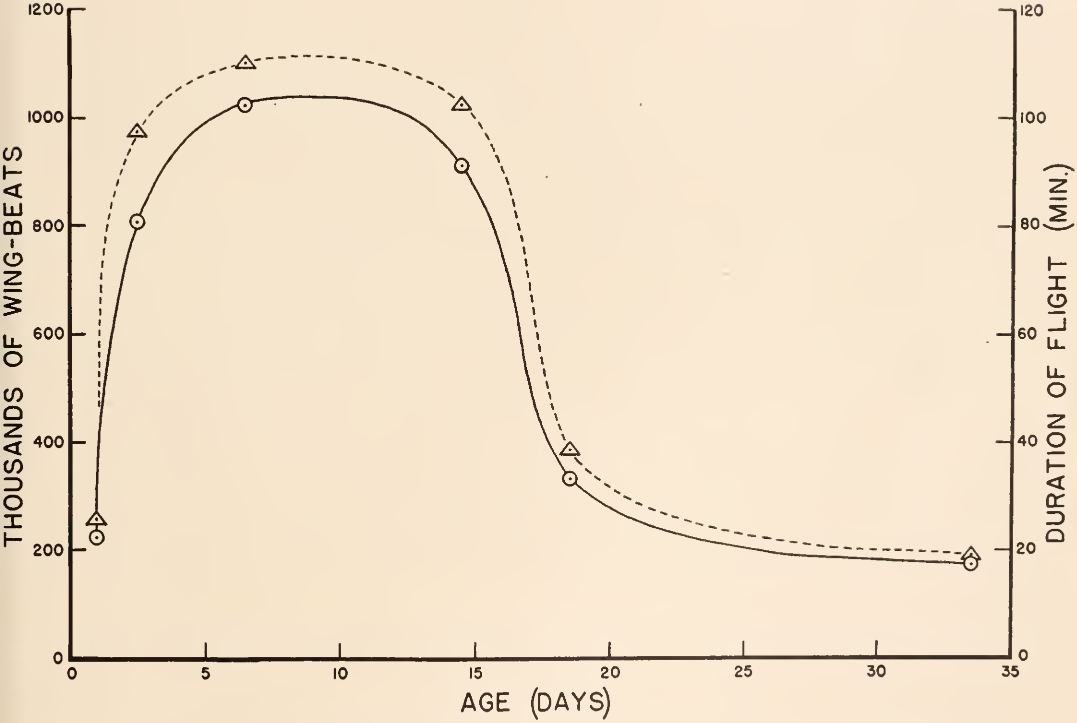

FIGURE 4. The relation between the flight ability and the age of Drosophila. Upper curve, average duration of flight as a function of age. Lower curve, average total number of wing-beats as a function of age.

It is clear that the flight ability varies markedly with age. Animals one to two weeks old can fly for an average period exceeding 100 minutes during which time the number of consecutive wing-beats is about a million. In contrast, one-day-old animals, or individuals older than 25 days, can fly less than 26 minutes (226,000 double wing-beats).

DISCUSSION

In view of the utilization of glycogen demonstrated in Figure 1, there can be little doubt that this substance is of primary importance in the flight of flies. The existence of a carbohydrate metabolism during flight has been previously indicated in investigations where the respiratory quotient of flying insects has been measured (Jongbloed and Wiersma, 1935; Chadwick and Gilmour, 1940). Although these studies demonstrated an R.Q. of unity, this value can also characterize protein metabolism, providing ammonia, carbon dioxide, and water are end-products.

It is a remarkable fact that the glycogen concentration that we found characteristic of *entire* Drosophila at the optimal age approximates the concentration reported for the liver of mammals at the optimal age; i.e., about 6 per cent of the wet weight (Heymann and Modic, 1939). Such high concentrations of glycogen were noted by Claude Bernard (1879), who stated in his description of the larva of the housefly (p. 114): "on peut dire, sans exagération, que ces larves sont de véritables sacs à glycogène."

Information concerning the role of glycogen in flight may be obtained by comparing the simultaneous changes in the glycogen concentration and in the frequency of wing-beat during continuous flights (Figures 1 and 2). Whereas the concentration of glycogen decreases regularly from the outset of flight to final exhaustion, the wing-beat frequency is essentially unaffected by this loss for a period after the initiation of flight. It thus appears that the neuromuscular system, in terms of the frequency of its activity, is in an approximately "steady state" during this period. The duration of this condition is apparently determined by the concentration of glycogen at the beginning of flight, for it is brief in animals that are either very young or very old and prolonged in animals at the optimal age of from one to two weeks. Hence, from this point of view, glycogen may be considered a reservoir of carbohydrate which is drawn upon during flight.

The experiments of Beutler (1936 a and b; 1937) on honey-bees are of interest in this connection. The length of flight was found to be determined by the concentration of sugar in the blood, and, furthermore, the concentration necessary for flight could be maintained for a prolonged period when the animal's "honey-bladder" was full. Since the amount of glycogen in the honey-bee is very low, amounting to only 0.3 to 1.0 per cent of the live weight, it is probable that in this animal the contents of the honey-bladder play a role similar to that of glycogen in flies.

There is evidence to discount the possibility that the stage of fatigue near the end of continuous flights in air results from the accumulation of lactic acid. As noted above, flight ability is not regained to any large degree after a rest of several hours following exhaustion. Furthermore, Chadwick and Gilmour (1940) have demonstrated that the oxygen debt of *Drosophila repleta*, following flight, is not more than 0.18 cu. mm. of oxygen, an amount which would be utilized in less than 6 seconds of flight. The magnitude of the oxygen debt likewise appeared to be independent of the length of flight. Hence the occurrence of fatigue is more adequately explained in terms of carbohydrate limitation than in terms of lactic acid accumulation.

The data presented in regard to the physiological ageing of Drosophila demonstrate that an optimum age exists for flight which, in general, coincides with the period of maximal glycogen content (Figures 3 and 4). The correlation between these factors is satisfactory, except that after reaching a maximum the flight ability decreases more rapidly with age than does the glycogen concentration. A possible explanation of this lack of complete agreement is the fact that entire animals were used in the analyses and, therefore, the percentage of glycogen that is unavailable for use during flight, due to its incorporation in the eggs and other tissues, could not be taken into account. The efficiency of glycogen utilization may also vary to some extent with age and thus affect the flight ability.

Nevertheless, the general agreement between the simultaneous changes in the glycogen content and in the flight ability strongly suggests that the former is causally related to the latter. If the maturation and senescence of the flight ability is thus explained, then the larger question arises concerning the identity of the factors responsible for the changes in glycogen content which occur as the animals grow old in the presence of an optimum environment.

SUMMARY

The role of glycogen in the flight physiology was studied for two species of flies, *Drosophila funebris* and *Lucilia sericata*. Glycogen was determined by microchemical methods. The flight ability was measured stroboscopically in terms of the total number of wing-beats, under standardized conditions, in continuous flights to exhaustion.

Glycogen was found to be of primary importance in the physiology of flight. During continuous flight the concentration of this substance gradually decreases in both the entire animal and the thorax.

The decrease in glycogen during the first stages of such flights has no marked effects on the intensity of flight, in terms of the frequency of wing-beat.

Near the end of continuous flight the concentration of glycogen becomes limiting and wing-beat frequency rapidly decreases until flight ceases before the frequency becomes as low as 100 double-beats per second.

Both the flight ability of Drosophila and the concentration of glycogen vary as functions of age. During the first week of adult life the average length of flight increases from 26 minutes on the first day to 110 on the seventh and the total number of wing-beats from 225,000 to more than a million. Simultaneously the glycogen concentration rises from about 2.5 to 6 per cent of the live weight. In animals older than two weeks the flight ability and glycogen concentration decrease rapidly and then more slowly until, by the thirty-third day, the average length of flight is reduced to 19 minutes (170,000 double wing-beats) and the glycogen concentration to about 3.5 per cent of the live weight. This correlation, although not exact, suggests that the physiological ageing of the flight ability results to a large degree from the simultaneous changes in the concentration of glycogen.

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METHODS OF ESTIMATING THE EFFECTS OF MELANOPHORE CHANGES ON ANIMAL COLORATION

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In animal chromatics melanophores have received more attention than any other form of chromatophore. This has been due mainly to the conspicuousness and relative permanence of their pigment and to the consequent ease with which the changes in the disposition of this pigment can be followed. The technique of recording such changes varies more or less with the investigator and may well be a subject for critical consideration. Such a consideration is the aim of this paper. The questions herein discussed are well illustrated by the color changes in the catfish, *Ameiurus nebulosus*, and this fish will be used as an example in much of the present discussion.

The older investigators in describing the various color conditions in animals were content to present them in terms of full paleness or full darkness as judged by the observer's eye. It soon became evident, however, that such gross distinctions were insufficient, and attempts were made to divide the color range of any given animal by points that would break it up into reasonable units. Many authors came to employ five such points which separated the whole range into quarters. The points thus used were, in addition to the extremes pale and dark, a middle point termed intermediate and two secondary points pale-intermediate and dark-intermediate in positions appropriate for these designations.

In judging by this method of the color condition of a particular fish at any moment it was found desirable to have fixed standard color samples for comparison. For such a fish as Fundulus these samples were easily made and preserved in formol-alcohol. Fixed samples of this kind are reasonably permanent and may be used with success in determining by ocular comparison the tints of living fishes in process of change. In Ameiurus a set of these samples has already been photographed and published (Figure 1). Ameiurus, however, offers by contrast an advantage over Fundulus in that four of its five stages in color change can be kept on hand conveniently and continuously as living laboratory material. The extreme pale state can be permanently maintained in fishes kept in white-walled vessels brightly illuminated from above (maximum effect of adrenergic fibers, very probably adrenaline; Parker, 1941). The extreme dark state, commonly called coal-black, is seen in blinded fishes also brightly illuminated (combined effect of intermedine and acetylcholine). Such fishes will remain completely dark even if kept with fully pale ones in white-walled, brightly lighted vessels. The only adverse effect in such a combination is to be seen in the pale fishes which will darken slightly in consequence of the presence of their coal-black neighbors as part of their environment. By a fortunate circumstance the intermediate state in Ameiurus is maintained by a hypophysectomized catfish with normal vision and in a black-walled lighted container (effect of cholinergic fibers—acetylcholine—alone). The dark-intermediate state is also a matter of coincidence in that it is seen in catfishes with normal vision and in a black-walled, well lighted vessel. Such fishes, contrary to what might be expected, do not become coal-black (Parker, 1941), but remain permanently in the dark-intermediate phase. The limitation of this response is not well understood. It may be determined by some peculiarity of the melanophores themselves or possibly by the absence of an additional dispersing neurohumor, for coal-black, which might be liberated on the loss of the

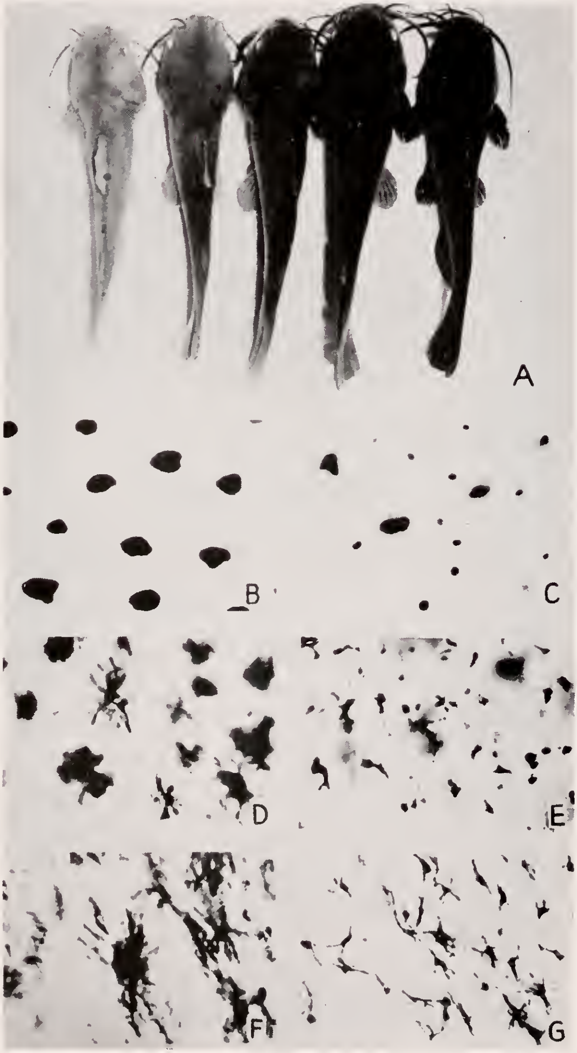

FIGURE 1. Range of tints from extreme pale to extreme dark (A) in the catfish Ameiurus and the conditions of its color-cells (B to G) at three stages. Macromelanophores (B) from derma and micromelanophores (C) from epidermis of fishes of palest tint showing maximum concentration of pigment. Large (F) and small (G) melanophores from coal-black fishes showing maximum dispersion of pigment. Conditions of the two kinds of melanophores (D and E) from fishes of intermediate tint.

eye. The last of these steps, the pale-intermediate, cannot be maintained easily in the living condition, but is best represented by a killed and preserved specimen. This phase doubtless could be established and kept in an illuminated vessel with walls of an appropriate gray, but this refinement has not been attempted. Thus four of the five critical steps in the color range of the catfish can be easily and permanently kept as shown in living laboratory examples for comparison with experimental fishes. Catfishes, the color states of which are to be determined, can be marked by the appropriate clipping of one of their fins and may then be liberated in a vessel with one or more standard fishes of given color for close comparison. Such comparisons yield surprisingly clear and definite results. Since the colors of catfishes are not immediately altered by handling and since the completion of their normal color responses to differences in the environment require hours or even days, comparisons such as those described, which take only a fraction of a minute to make, may be carried out with security. Tests of this kind are avowedly crude, but the terms in which they are described are not inexact as stated by Waring (1942). It would be impossible for any one working in animal chromatics to proceed far without the use of precisely such methods of ocular comparison as those described, methods which have served as the basis of much recent work on animal chromatics.

It was a natural step as the subject of animal color changes developed for workers to seek the relations between the color states of a given animal and the conditions of its chromatophores, particularly of its melanophores. The extreme conditions of the melanophores in the fully pale and fully dark Fundulus were photographed as early as 1913 by Spaeth. In consequence of the form assumed by the melanophore pigment in the two extremes and the intermediate tint of animals, it became usual to designate these pigment shapes as punctate, stellate, and reticulate. The states of the melanophores at the quarter points in the animal's coloration were called by some workers puncto-stellate and reticulo-stellate. Thus this method though accurate in its way grew to be cumbersome in its nomenclature (Waring, 1942) and it is not surprising that it failed to gain great favor.

A closely related treatment of the total melanophore range was put forward in 1928 by Slome and Hogben. It has since been several times redescribed and somewhat elaborated (Slome and Hogben, 1929; Hogben and Gordon, 1930; Hogben and Slome, 1931; Waring, 1942), and is now much in use. It consists in an arbitrary division of the whole melanophore range into four stretches by five division points which correspond very closely to the five points designated in the older nomenclature as punctate, stellate, reticulate, etc., and in giving to each of these five points a numerical designation from 1 for punctate to 5 for reticulate. The states of concentration or of dispersion of the melanophore pigment for the five points have been illustrated by sketches (Hogben and Gordon, 1930; Hogben and Slome, 1931) and these sketches have served as definitions for the points. This method at once did away with the cumbersomeness of the older terminology and gave to the work in this field not only greater convenience but a certain quantitative aspect. By means of this system melanophore indices could be established for the several states of the color-cells which could then be plotted against time so as to allow a graphic representation of the changing melanophores. Such plottings have been very freely employed by recent students of color changes

(Hogben and Landgrebe, 1940; Waring, 1940; Neill, 1940) and have yielded interesting and important results. Several modifications of this system have been offered. Following the procedure introduced by Hewer (1926) Matsushita (1938) distinguished in the melanophore pigment changes from full concentration to full dispersion of the Japanese catfish Parasilurus six instead of five steps. These steps were defined by means of accurately drawn illustrations (Figure 2). By the use of a simple formula Matsushita obtained indices of the average conditions of the melanophores in a given fish at different color phases and plotted these on a scale of one-hundred against time. Thus this worker arrived at an exposition of his results much like that employed by Hogben and others, but on the basis of finer gradations. A move in the opposite direction was taken by Sawaya (1939) followed by Mendes (1942) both of whom, like Hogben and his co-workers, distinguished five dividing points in the melanophore scale, but numbered them in reverse order, I for maximum dispersion and V for maximum concentration. In the plottings made by Sawaya no averages were employed nor were curves drawn as was done by Hogben and his associates. In consequence Sawaya's tables show the coarseness of his original observations and lack much of the detail shown in the plottings by Hogben and his school. If in refinement Matsushita has somewhat overdone Hogben's method, Sawaya has on the whole underdone it.

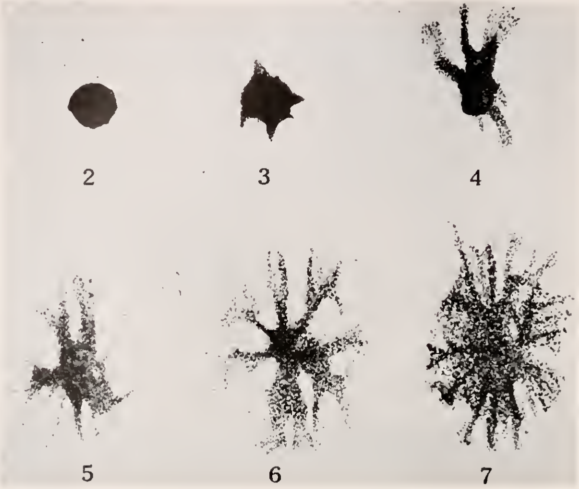

FIGURE 2. Six phases in the changes of a melanophore from its punctate condition (2), through its stellate stage (4) to its reticulate state (6, 7). The corresponding color conditions in the fish are pale (2), intermediate (4), dark (6), and coal-black (7). From the Japanese catfish *Parasilurus asotus* (L.). Matsushita (1938).

The replacement of descriptive terms for the states of melanophores from punctate to reticulate by numbers has not only added great flexibility to the treatment of color changes, but, as already stated, has given the subject a quantitative aspect. This, however, may be its gravest defect, for it has tempted some of the less critical workers in this field into too great a reliance on what may be done with the quantitative statements that it has been brought to yield. The originators of this method repeatedly called the attention of those who might use it to the fact that the subdivisions whereby the steps in the melanophore changes are indicated are made on an arbitrary basis which means that 4 in the scale series is not necessarily twice 2, nor 5 five times 1. Under these circumstances it is very questionable how legitimate are the averages and other mathematical results that have been indulged in and the reliability of the curves based upon these results. It seems possible that to a certain extent the method has run away with its proponents. Undoubtedly it can be made to lead to conclusions of much value, but it must be used with restraint, probably with much more restraint than has been exercised by some of its very recent advocates. No better caution as to its use can be given than that contained in the following passage from the paper in which the method was described by Slome and Hogben (1929). The authors of this paper remark concerning plottings, etc., based upon the use of this method that "in interpreting these results, which are presented in graphic form, it must be borne in mind that the numerical symbols applied to different configurations of the dermal melanophores are quite arbitrary, and therefore, though some insight may be obtained from a consideration of the intervals which elapse between equilibrium conditions and the intercalation of subnormal or supranormal phases, no significance can legitimately be attached to the gradients of the curves." So clear and understanding a caution as this calls for more conservative estimates of results than those that have been proposed by some of the more recent workers. The temptation seems to have been to use such quantitative results as though they were founded on solid measurements instead of on arbitrary assignments. Because of the tempting ease with which reasonable boundaries in this kind of work can be overstepped, one is led to see greater real security in Sawaya's coarser system or even in the earlier one of cumbersome adjectives for melanophore gradations which are only in a remote way suggestively quantitative. Possibly such a descriptive system may be as a matter of fact more truthful in portraying what is really observed about color-cells than one based on arbitrary numerical units not soundly quantitative.

From time to time systems for the recording of chromatophores much more firmly grounded than that introduced by Slome and Hogben (1928) have been suggested. One of these advanced by Spaeth (1913b, 1916) much antedates that by Slome and Hogben. Spaeth discovered by following a line of work initiated by Ballowitz (1913) that the living melanophores in the freshly removed scale of Fundulus could be made by an appropriate treatment with barium chloride and sodium chloride alternately to disperse and to concentrate their pigment. This type of response which was rhythmic in character was at a rate essentially the same as that of the normal color change. Such rhythmic pulsations of the color-cells reach from complete concentration to complete dispersion and thus reproduce normal melanophore activity. By means of an ocular micrometer the changing diameter of the pigment mass in a single color-cell can be measured step by step, and the records thus obtained can be plotted against time as a graphic description of the activity of the melanophore. Thus some thirty pulses of a single color-cell were plotted by Spaeth over a period of about an hour. Spaeth (1916) subsequently changed his method and rendered it somewhat more mechanical by adding to his microscope a recording device by which the tip of the column of pigment could be followed as it advanced into the process of the color-cell or retreated from it. This gave almost perfect time records of the activity of the melanophore on the basis of absolute measurements. The method seems to have attracted no attention for it appears not to have been used nor criticized.

A second largely objective technique for measuring melanophore activity was devised by Hill, Parkinson, and Solandt (1935). These workers threw a constant beam of light on the back of a restrained Fundulus the surroundings of which could be altered from black to white or the reverse thus to induce the fish to change color. The light reflected from the illuminated spot on the back of the changing fish was focussed on a photoelectric cell and the steps of change read off in a galvanometer. Thus measurements were obtained that could be plotted against time, and in this way curves for the dispersion and the concentration of melanophore pigment could be obtained. This method agrees with Spaeth's in that it is based upon absolute units. It has been criticized by Wykes (1937) and by Neill (1940) who object to it on the ground that it gives the "sum effect of color response only and . . . no information as to the activity of different pigmentary effectors." From the standpoint of its general applicability this is a serious defect. It must be borne in mind, however, that the fish used by Hill and his associated, *Fundulus heteroclitus*, has on its back whence the reflected light was taken very few chromatophores except melanophores. The scattered xanthophores in this part of its body are insignificant in comparison with the dark color-cells. Consequently the measurements recorded by Hill and his co-workers from this part of the body of Fundulus are almost entirely dependent upon melanophores. Of course in a fish such as Ameiurus where only melanophores are present Wykes' criticism does not apply.

A third distinctly objective method for the study of melanophore changes is that devised by Smith (1936). This method like that of Spaeth depends upon the use of pulsating dark color-cells in freshly removed scales, in this instance from the fish Tautoga. A beam of light is thrown through such a scale under the microscope and the change of intensity in this light as determined by concentration or dispersion of the melanophore pigment is read off by a combination of a photoelectric cell in the microscope and an outside galvanometer. By this means readings can be taken at 10-second intervals or from ten to 15 readings for a single chromatic pulse. These readings can be plotted against time and thus made the basis of a curve for chromatophore activity in the same way as in Hill's method. The chief difference between Smith's method and that of Hill is that whereas in Smith's technique transmitted light is measured in Hill's it is reflected light. Smith's method like Hill's is based on absolute measurements. It is also open to the same criticism as that urged by Wykes against Hill's procedure. But this has as little force in the case of Smith's records as it had in those of Hill, for in Tautoga, the fish used by Smith, the coloration of the scales is due predominantly to melanophores. At the outset of any tests the melanophores in Tautoga commonly beat in phase, which as Smith pointed out, is essential to good readings. In course of time, however, many of them drop out of step with the result that the records, for instance, of the second quarter of an hour are less regular than those of the first quarter (compare Figure 1, Smith, 1936). Notwithstanding this defect Smith's method has yielded the clearest and most convincing plottings of melanophore activity thus far published.

The last three methods here discussed, those of Spaeth, of Hill, and of Smith, are all based on sound physical measurements either of length or of light intensity. In this respect they are much superior to that of Slome and Hogben whose proposed units are arbitrary and lack real substantiality. As Slome and Hogben themselves remark, "no significance can legitimately be attached to the gradients of the curves" obtained by their technique. Such is not true of the records of the last three methods here described. These, but particularly the methods of Hill and his associates and of Smith, show curves that are consistently uniform and characteristic. These curves are S-shaped, sigmoid in form. This form of curve was noted by Parker (1935) in a discussion of the color changes in Fundulus. The color changes in this fish are almost entirely under nerve control. Blanching begins slowly due to the gradual accumulation of a concentrating neurohumor, probably adrenaline from adrenergic fibers, in the fluids around the melanophores. The later rapid increase of blanching indicates a high concentration of this paling neurohumor, and the following decline in the rate of color change till it reaches full cessation marks without doubt the limit of responsiveness of the melanophores to the activating agent. Darkening in this fish follows a similar course but in reverse direction and is probably due to the nervous neurohumor acetylcholine, for intermedine appears to play little or no part in this phase of Fundulus. The sigmoid form of the curves for melanophore activity is especially well shown in Smith's plottings, but it is to be inferred clearly and easily from those by Hill and his associates. It can be discerned even in the graphs made by Slome and Hogben's method though the fact that the plottings based upon this technique usually begin at what is the middle of such a curve disguises the whole reaction measurably. Nevertheless the elements of such a curve are there discernible. Thus the normal change in the dispersion and the concentration of melanophore pigment in a number of fishes appears to conform, when plotted, to a type of curve, the sigmoid curve, which is characteristic of the course of many living processes.

In such a fish as Fundulus where the predominant chromatophores are melanophores or in Ameiurus where the color-cells are exclusively of the dark type the color changes conform very exactly to the states of the color-cells. In dark fishes the melanophore pigment is greatly dispersed, in pale ones greatly concentrated. This position has been opposed by Neill (1940) who has contended that the color of a given fish is not closely related to its dark cells and he has tabulated conditions in the eel to substantiate his contention. As the foregoing discussion shows, a determination of this kind depends upon the chromatophoric constitution of the given fish. In the catfish with only melanophores the agreement is as near exact as can be measured, but in the eel with a sexually variable skin-background and several classes of diverse chromatophores it is not to be expected that there would be full agreement between the general tint of the fish and one set of color-cells, the melanophores. It is surprising indeed that, as the table published by Neill shows, the agreement in the eel is so close. That general color and states of melanophores are as intimately related as they are in many fishes indicates that of the various types of color-cells the dark ones commonly predominate and consequently the color changes follow in the main this type of chromatophore. In work of this kind anyone who wished to investigate the activity of xanthophores would not choose a fish whose color-cells were predominantly melanophores.

Another question in dealing with melanophores has to do with the means by which the momentary state of a changing dark color-cell is to be recorded. For this purpose photography has been of service. By means of succession photographs of the same living melanophores at different stages the changes in these color-cells have been followed in small groups (Spaeth, 1913a) in a single cell (Perkins, 1928), or in a larger group (Parker, 1935). This procedure calls for the repeated identification in a living animal after considerable intervals of time of a particular color-cell or group of such cells and their rephotographing, an exacting exercise at the least. Moreover the handling of some live fishes induces under certain circumstances changes in the states of their color-cells that are disturbing in such an operation. Thus Fundulus darkens noticeably when taken from the water and handled. It is therefore not surprising that this method is not in common practice, yet it has yielded significant results in the study of the diffusion of neurohumors (Parker, 1935).

The great difficulty in determining the exact condition of melanophores in living fishes, as might be inferred from what has been stated, is the ease with which many creatures respond by melanophore changes to handling and the like. This capacity is very different in different species. Thus in the catfish scarcely any change in color at all is to be seen on reasonably mild manipulation. Flatfishes on the other hand are very responsive to the slightest environmental disturbance such as a tap on their container or even the passage of the hand over the aquarium in which they are kept. Sticklebacks, according to Hogben and Landgrebe (1940), are moderately susceptible to such shocks and may thus be brought to shift their tints toward an intermediate phase if in the beginning they are at either extreme of color. To avoid these disturbing drifts Hogben and Landgrebe put single sticklebacks each in a small glass vessel supplied with a suitable current of water and with apertures by which the fish could be introduced and through which its tail could project. In taking readings such a glass with its contained fish was removed from the general aquarium, and with the fish's tail projecting quickly but under the microscope with the tail in the field. Records were then made of the states of the melanophores and the fish discarded, for experience showed that it was not favorable material for further work. Much the same technique was followed by Neill (1940) in his study of the color changes of the eel and other fishes. It has long been the practice in the Harvard Laboratories to treat Fundulus in this way, but the color responses of this fish on handling take place so quickly that only approximate records can thus be obtained and these can be used only as rough indications of what is happening.

To permit of deliberate inspection and measurement of melanophores under the microscope permanent preparations of the tails and fins of fishes have been made. Such preparations were prepared and photographed as early as 1934 by Parker. The method has also been employed by others especially by Wykes (1937). Much of its success depends upon the way in which the fins have been prepared, and as this technique is nowhere adequately described, the following brief account of it is given.

Permanent preparations of the fins of catfishes can be easily and quickly made by the following steps. With a strong pair of shears the fish is decapitated and its caudal fin severed from its body. This fin is then at once pinned out under water on a broad wooden spatula. To make a smooth preparation the fin must be fully stretched on the flat surface of the wood and held there firmly by the pins. The position of the two pins used for this purpose are shown in Figure 3 by the two holes, one each in the upper and lower margins of the tail near its root. As soon as the fin is stretched on the spatula the fin and spatula together are dipped momentarily in water at 60° C. This kills the tissues of the fin at once and thus stops any possible change in its melanophores. Such a method, which is the one used by Wykes (1937) and by me, is much quicker and therefore much more reliable than that employed by Waring, namely, fixation in Bouin fluid which, though a rapid killing agent, is by no means so rapid as heat. From the hot water the spatula and its attached fin are then transferred to a preservative such as formaldehyde-alcohol where they should remain about half a day. The preservative regularly used in this work was a mixture of equal parts of 95 per cent alcohol and 10 per cent aqueous formaldehyde. The success of the operation thus far depends upon rapidity. The period from the moment the fish is decapitated till its fin is histologically fixed by hot water must be as short as possible. With practice this interval can be reduced to some 25 seconds. So short a time is of no significance for the pigment movements in many fishes where, as in the catfish, the pigment change is a matter of hours or even days. The technique here described would of course be useless for a species such as the squirrel-fish whose change of pigment may be completed in as short a time as five seconds. In fishes the rate of whose color change is moderately slow, however, the method has proved to be fully satisfactory.

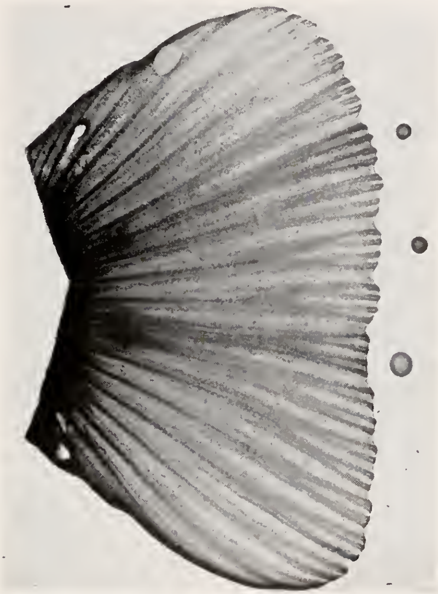

FIGURE 3. Preparation of the tail-fin of the catfish Ameiurus with two caudal bands. The upper band is partly blanched but not as much so as the rest of the tail. The lower band was cut at the same time as the upper one and blanched at the same rate as that one did. Before the preparation was made the lower band was recut whereupon it darkened as compared with the upper band. Parker (1943).

From the formaldehyde-alcohol mixture the spatula with its attached fin is next transferred to 70 per cent and then to 95 per cent alcohol. After hardening in this stronger alcohol for half a day the fin may be unpinned from the spatula, freed of its superfluous muscle, etc., and put into absolute alcohol. When dehydration is complete it can be cleared in xylol, and mounted in xylol-balsam on a large slide under a cover-glass. Preparations of this kind may be conveniently used for study under the microscope and for photographic purposes (Figure 3). In such a preparation caudal bands of different states may be studied and compared. Even to the unassisted eye such bands may be characteristically different. Thus in the prepared tail shown in Figure 3 the uppermost band, induced by cutting the ray opposite the uppermost dot, was made several days before the fin was prepared. This band being on a pale fish gradually blanched, but not as much as the normal rays did as can be seen by comparing the ray opposite the middle dot with the one in question. The lower band opposite the lowest dot was made at the same time as the upper one, but after it had blanched to the same degree as the upper band it was recut with the result that its dark tint was revived. The revival of such bands by recutting is a matter of first importance in questions of color change and such a preparation as that shown in Figure 3 demonstrates how certain in their results materials of this kind are even to the unaided eye. Under the microscope melanophores in such preparations can be measured with a deliberate accuracy that no other method permits.

This method has been criticized by Neill (1940) on the ground that the preparation of the fin alters the form of the pigment mass so as to distort the record. According to Neill a melanophore index may change as much as a fourth of the whole scale due to the process of preparation. Such changes may possibly take place. If they do, they depend upon the rate at which the preparation is made as compared with the rate of color change in the given fish. Not the least indication of such a disturbing change is to be seen in the preparation of catfish fins. These fins can be prepared in less than half a minute whereas the color changes in this fish require hours or even days. Even in Fundulus where the color change is accomplished in a few minutes serviceable preparations can be made by this technique though this is perhaps the most rapid fish that can be used in this way. For fishes with a slower rate of change, and there are many such, the method is an admirable one. To such fishes the criticism advanced by Neill does not apply. In general I agree with Wykes (1938) when she declares that "microscopic examination of the state of the melanophores before and after fixation showed that the fixative had no detectable effect on the condition of these cells." Neill's criticism of this technique is certainly of most limited application. A real objection to this technique, however, is that it necessitates the death of the fish and hence prevents a continuous series of observations over the whole range of a color change. But this is no worse than what is necessitated by Hogben and Landgrebe's method where after one record the fish for the sake of security in later records is discarded.

Wykes' method of dealing with the melanophores in fixed preparations was to measure as did Spaeth (1913b), the diameter of the area covered by each of a large number of color-cells, average these measurements, and accept the average thus obtained as a rating for the melanophores of the given preparation. Such ratings could then be tabulated or, better, plotted against time and thus a graphic picture of the particular melanophore change could be obtained (Wykes, 1937).

Such technique has been applied with success to the catfish. In this animal as is well known there are two sets of melanophores, micromelanophores in the epidermis and macromelanophores in the derma (Figure 1). In the concentrated state the micromelanophore pigment mass (Figure 1, C) has a diameter of about 12 microns, the macromelanophore mass (Figure 1, B) of about 45 microns. Reverting momentarily to the descriptive nomenclature for chromatophore states the punctate condition of the macromelanophore has then a diameter of about 45 microns. The diameter of its stellate condition is approximately 100 microns (Figure 1, D) and of its reticulate state 145 microns (Figure 1, F). Thus the difference between the extremes in the diameter of the pigment spread in the macromelanophores of catfishes is roughly 100 microns and the average diameter at their maximum is some three times what it is at their minimum.

These quantitative statements give much that is illuminating in the study of catfish melanophores, but in this particular animal they have a marked insufficiency. For instance, they omit a very important and significant feature in the dark phase of catfish economy. To the human eye the dark phase of this fish so-called, that seen in a normal catfish in an illuminated black-walled vessel, and the coal-black phase shown by it in the eyeless condition are easily distinguishable (Figure 1, A, extreme right-hand fish and its immediate neighbor; compare in particular the tints of the fins). Yet the melanophores of these two phases cover similar areas. They would both fall under the same index, number 5, of Hogben and Slome; their diameters would be the same as measured by the method of Wykes. The fishes differ clearly to the unaided eye yet their melanophores would not differ by the methods of Hogben and Slome, and of Wykes. Where they are unlike is in the spread of their processes particularly in the regions of the roots of these processes (Compare Figure 2; 6 and 7). It is these heavier roots rather than a difference in total area covered by the melanophore that gives the coal-blackness to the blinded fish as contrasted with the mere darkness of the seeing one. Thus coal-blackness is a feature more easily recognized by the unaided eye than it is by other methods, a circumstance which points to the importance of the total inspection of color tints.

Herewith is concluded this survey of the more important lines of technique whereby connections are sought between the color changes in animals and their chromatophores especially their melanophores. The conclusion to be drawn from this survey is that animal color changes and their color-cells are so diversely and intricately related that no single method is adequate as a means of complete elucidation. For one species a particular technique is more favorable than for another. Even the older methods of color comparison by the unaided eye when properly carried out yield results that are surprisingly worth while. Thus far adequate quantitative results have scarcely been attained, for much of the work done on the basis of arbitrary units will require revision and such quantitative technique as is really soundly reliable has not yet been put into conveniently workable form. A thoroughly serviceable quantitative technique for the study of color changes and their underlying mechanism is still to be devised. Meanwhile none of the several methods adopted by different workers can well be ignored, for notwithstanding the broad condemnation issued by such workers as Neill (1940) and Waring (1942) for all methods except their own, no single method has such superiority over others that it can enjoy exclusive possession of the field.

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